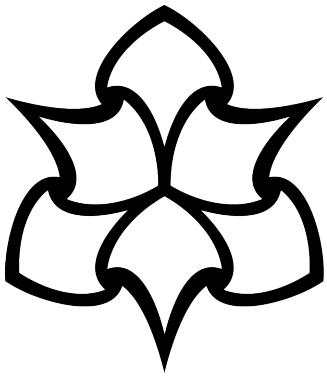

INTEGRATIVE PHYSIOLOGY OF HUMAN AEROBIC FITNESS AND THE INFLUENCE OF THE ACE I/Dp GENOTYPE

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PhD **2013**

INTEGRATIVE PHYSIOLOGY OF HUMAN AEROBIC FITNESS AND THE INFLUENCE OF THE ACE I/Dp GENOTYPE

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Chapter 1

List of abbreviations

AA	Arachodonic Acid
ACE	Angiotensin Converting Enzyme
ACE I/Dp	Angiotensin Converting Enzyme Insertion/Deletion polymorphism
ACEi	Angiotensin Converting Enzyme Inhibitor
ATP	Adenosine Triphosphate
AGN	Angiotensinogen
Ang2	Angiotensin 2
AT1R	Angiotensin Type-1 Receptor
AT2R	Angiotensin Type-2 Receptor
ATR1B	Angiotensin Type-1 Receptor Blocker
BCAA	Branched Chain Amino Acid
BK	Bradykinin
BK2R	Bradykinin Type-2 Receptor
BP	Blood Pressure
CAD	Coronary Artery Disease
Cd	C-domain
CHD	Coronary Heart Disease
CHO	Carbohydrate
CVD	Cardiovascular Disease
CPT-1	Carnitin Palmitoyltransferase I
CPT-2	Carnitine Palmitoyltransferase II
CVS	Cardiovascular System
CYC1	Cytochrome c Oxidase
DM	Diabetes
DM1	Diabetes Type 1

DM2	Diabetes Type 2
DZ	Dizygote
EC	Endothelial Cell
EDHF	Endothelial Hyperpolorising Factor
ET	Endothelin
ETC	Electron Transport Chain
FGF	Fibroblast Growth Factor
FMD	Femoral Mean Dilatation
FSS	Fluid Shear Stress
HIF-1	Hypoxia Inducible Factor 1
HUVEC	Human Umbilical Vein Endothelial Cells
IMTG	Intramuscular Tricylglyceride
LDL	Low Density Lipoproteins
LPL	Lipoprotein Lipase
LSS	Laminar Shear Stress
MZ	Monzygote
Nd	N-domain
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
O_2	Oxygen
QOL	Quality of Life
RAS	Renin Angiotensin System
RBC	Red Blood Cell
RT-PCR	Real Time - Polymerase Chain Reaction
SINE	Short Interspersed Elements
TAG	Tricylglyceride

TE	Transposable Elements
Tfam	Transcription Factor A, Mitochondrial
TnC	Tenascin-C
VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth Muscle Cell

Publications (see Appendix 2)

- Martin Flueck, **David Vaughan**, and Hakan Westerblad. Linking genes with exercise: where is the cut-off? European Journal of Applied Physiology (2010): 110; 1095-1098.

Manuscripts submitted and in preparation

- **David Vaughan**, Felicitas A Huber-Abel, Franziska Graber, Hans Hoppeler, and Martin Flueck. The Angiotensin Converting Enzyme Insertion Polymorphism Alters the Angiogenic Response of Skeletal Muscle to Exercise. (Submitted to the European Journal of Applied Physiology, on February 10th 2012).
- **David Vaughan**, William Allwood, Felizitas Huber-Abel, Hans Hoppeler, Joern Rittweger, Royston Goodacre, Martin Flueck. Angiotensin Converting Enzyme Exerts System Control Over Fuel Handling In Skeletal Muscle. (In preparation, modified from data presented in chapters 3 and 4).

Conference proceedings

- Michael Brogioli, **David Vaughan**, Wouter Eilers, Sarah Waldron, Martin Flück, Glucose oxidation during endurance work is reduced in ACE genotypes lacking the I-allele, 4. Congress of the Sportwissenschaftliche Gesellschaft der Schweiz (2012), Eigenössische Hochschule für Sport, Magglingen, Switzerland.
- M. Flueck, **D. Vaughan**, W. Allwood, H. Hoppeler, F. Huber- Abel, J. Rittweger, W. Dunn, S. O'Hagen and R. Goodacre. Angiotensin converting enzyme exerts system control over fuel handling in skeletal muscle, Experimental Biology (2011), Washington DC (USA), D636 862.5.
- **David Vaughan**, William Allwood, Hans Hoppeler, Felizitas Huber-Abel, William Dunn, Stephen O'Hagen, Royston Goodacre, and Martin Flueck. Angiotensin Converting Enzyme Exerts System Control Over Fuel Handling In Skeletal Muscle. University College Dublin (2010). Proceedings of The Physiological Society 19, PC175. Poster Communications.
- **David Vaughan**, Hans Hoppeler, and Martin Flueck. Aerobic fitness in humans is under system control by the angiotensin 2 pathway. University College Dublin (2009). Proceedings of The Physiological Society 15, C34. Oral Communications.

Abstract

The search for genes that influence human performance and health constitutes a popular topic of current research. One such genetic constituent that has caused much interest over the last 20 years is the angiotensin converting enzyme insertion/deletion polymorphism (ACE I/Dp). There is much controversy in the literature regarding the role (if any) of this polymorphism as effects and effect size vary between populations of different origin and training status. The aim of this thesis was to analyse at the whole organism level whether skeletal muscle plasticity explains the association of the ACE I/D polymorphism with metabolic fitness.

Regular endurance exercise reduces the risk of a plethora of diseases, but the exact molecular mechanisms are not fully understood – the ability of muscle to adapt to exercise stimulus is key. Trained individuals demonstrated clear physiological differences of aerobic processes such as increased oxygen usage, greater power output and reduced body fat that would be expected (T-Test: $p<0.001$). By contrast, when examining metabolite changes, at rest, in the local muscle there were few ($n=6$) non-polar (lipid species) metabolite (assessed by mass spectrometry) differences between the trained and untrained. However, after an acute exercise bout working muscle in trained individuals displayed a significant up-regulation of ($n=76$) non-polar metabolites (Repeated ANOVA: $p=0.0004$), illustrating that training produces significant adaptations in substrate metabolism at the local level.

Would there be a genetic component contributing towards these physiological and local muscle differences? Individuals with the ACE I-allele (insertion sequence) had increased capillary density, and there were significant differences in transcripts, together with both polar and non-polar metabolites in the untrained population at rest and following an acute exercise bout. These differences were lost in the trained population. In a different population (Swiss) capillary density was increased following a training programme in the absence of the I-allele – in contrast to the other population (British). However, gene expression response of important factors, to exercise was preserved.

In conclusion, a trained population demonstrated enhanced non-polar metabolism in the working muscle after an exercise bout, and the dominant stimulus of regular exercise over-rides the influence of the ACE I/Dp. Nurture over-rides nature.

Chapter 1

Literature review;
Skeletal Muscle, the Cardiovasculature, Physical Activity and the Role of the ACE I/D Genotype

1 Literature Review: Skeletal Muscle, the Cardiovasculature, Physical Activity and the Role the ACE I/D Genotype

1.1. Skeletal muscle: Importance for health and disease

The evidence for the multitude of health benefits attributed to physical activity is now without question. Physical activity not only greatly improves an individual's quality of life (QOL) but also significantly reduces the economic burden to society as a whole, (POST, 2001; WHO, 2001, 2003; DoH, 2004, 2005). Despite solid evidence of positive health benefits, physical activitys importance, in both prevention and treatment of diseases, is under-valued and poorly communicated. Possibly one of the fundamental reasons regarding this "lack of interest" to embrace and acknowledge physical activity as a genuine intervention (in the management of disease) is the paucity of concrete evidence that details exactly how it confers health benefits (Coffey & Hawley, 2007).

In the post-industrial world (within the last 75 years) inactivity-related diseases have increased in frequency: diseases of "lifestyle", chronic diseases such as coronary heart disease, diabetes, stroke, obesity, osteoporosis and certain cancers (to name the main ones). The risk of developing one or more of the first four listed increase significantly with a concomitant decrease in physical activity levels (Khaw *et al.*, 2008). These four diseases are all related to a malfunctioning metabolism, where too little physical activity coupled with excessive energy consumption have become manifest in a disease phenotype. These metabolic diseases are associated with increased relative risk of disability, reduced QOL and even mortality (Rao, 2005; Butland *et al.*, 2007). A much under-valued organ that plays a significant role in the development and (potential) prevention of these metabolic diseases is muscle. However, the exact function of muscle in the aetiology of these diseases is undervalued (Vaag, 1999; Booth *et al.*, 2002) and not understood. It is known that muscle phenotype (visible traits) is conditioned by nurture (e.g. environment and behaviors), but exactly how does this occur and how does physical activity influence the way in which muscle responds and adapts (plasticity)? There is a shortage of scientific information on signaling mechanisms, coupled with how they relate and integrate through different system levels, which produce adaptations in muscle. Figure

1 illustrates the different system levels with an intact whole-organism (human). Substrates, including fat, carbohydrate (CHO) and to a lesser extent, proteins, are used to derive the energy needed for muscle contraction. The metabolism of these substrates can converge on the mitochondria to produce the body's main energy molecule, adenosine triphospate (ATP). This process does not only provide the ATP to enable muscle contraction, but also by-products such as urea from protein degradation, water and carbon dioxide from CHO and fat metabolism, and heat.

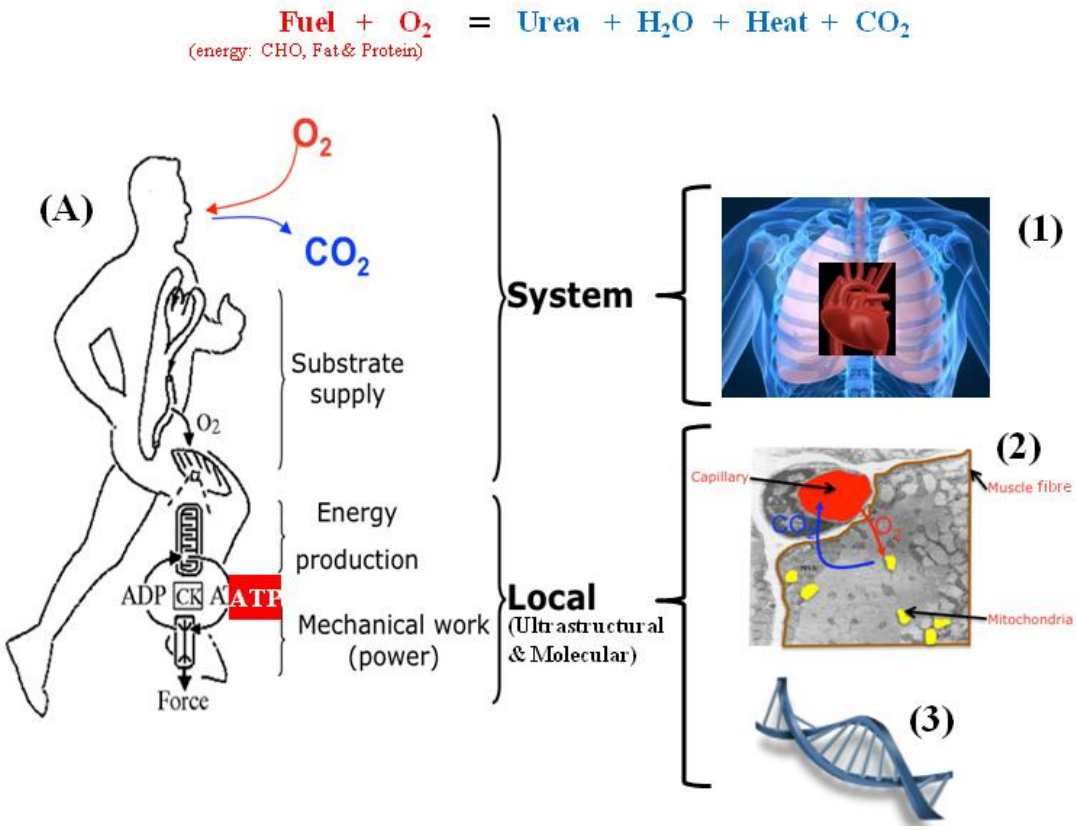

Figure 1. Different system levels in an intact whole-organism.

(1) Heart and Lungs represent the key organs involved in extraction and delivery/removal of O_2 and CO_2, (2) Ultrastructures involved in delivery/removal (capillaries), energy production (mitochondria), and movement (muscle fibre) – image provided courtesy of Hans Hoppeler, (3) Genes represent the "bottom" level in the whole system, which provide the blueprint for all the proteins required for physiological functioning. (A) Illustration from Linstedt & Conley (2001).

Even though we (humans) are all extremely similar, e.g. we all belong to the same family of *Homo sapiens* (modern human lineage), physiologically and genetically there are small yet significant subtle differences between each of us, which result in vastly different phenotypes. Even genetically identical twins will adapt differently,

right from gene regulation/expression to physiological adaptations. Therefore it is not the genetic make-up that is crucially important, but something else. Rennie highlighted that muscles response (Figure 2) to differing types of exercise (endurance or resistance) produced completely different muscle phenotypes (Rennie, 2005). The pertinent point highlighted was that the muscle response was not due to different genetic differences, as the two humans pictured are genetically identical twins (Figure 2).

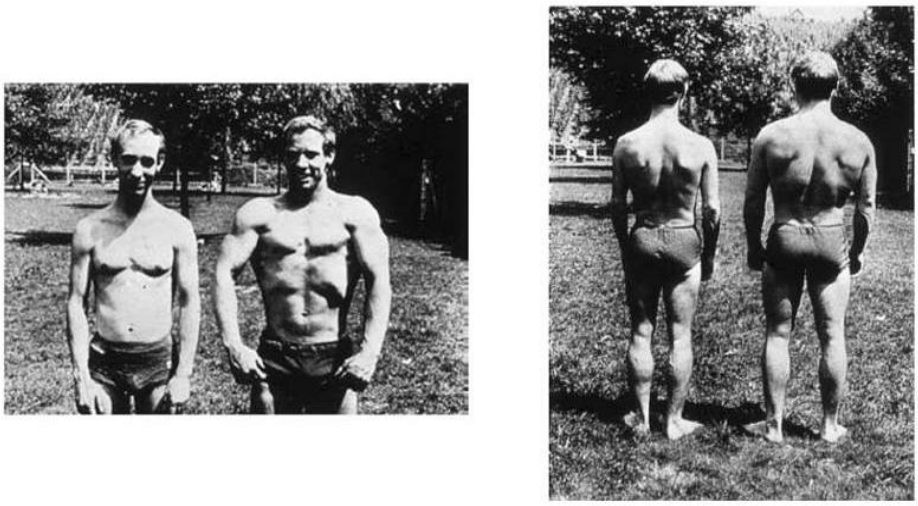

Figure 2. Genetically identical twins, aged 23, July 2nd 1969.

Both elite athletes, one an endurance runner the other a strength/field athlete (guess which is which!). Both images are from Rennie (2005).

1.1.1 Skeletal muscle and the vascular system

It is now well recognised that our modern lifestyle (energy rich diet and physical inactivity) is associated with an increased prevalence of chronic diseases. These well documented and studied chronic diseases (cardiovascular disease (CVD), obesity, DMII, stroke, certain cancers, and osteoporosis) are mainly the result of a malfunctioning metabolism of which the cardiovascular system (CVS) and muscle play a critical role in. The major cause of morbidity and mortality in modern society is coronary heart disease (CHD), of which hypertension is the number one risk factor for developing CHD (Dickson & Sigmund, 2006). The risk of becoming hypertensive (>140/90 mmHg), in modern societies, during a lifetime surpasses 90% (Messerli *et al.*, 2007). Hypertension cost the US economy in the late 1990's $286.5 billion (Booth

et al., 2000). The cost to the European economy of CVD in 2006 was just under €110 billion (BHF, 2009). It is not just the loss of human life or QOL that is concerning governments across the globe. The American Heart Association (AHA) estimates that one-third of the US adult population is hypertensive, which includes a third that is undiagnosed (Dickson & Sigmund, 2006).

Muscle can be thought of as a metabolic sink (Hamilton & Booth, 2000), an organ that can "absorb" or utilise substrates. Muscle produces movement, but this is only sustainable (past very short bursts, e.g. you can run 100 metres whilst holding your breath: glycolysis) with an adequate supply of oxygen (O_2) and energy substrates, plus the removal of carbon dioxide (CO_2) and other waste products. This is the role of the CVS or vasculature, which perfuse (to varying degrees) all tissues. The heart provides the force and the vasculature the conduits through which these metabolites are delivered (and removed) to the various tissues. The distribution of blood through the body, and resulting change with physical activity is illustrated in Figure 3 (Astrand, 2003).

Depending on the activity (sitting, eating, sleeping or physical activity) the CVS is constantly responding to changes. Effeciently regulating blood flow between different tissues and activity means many levels of control are required. At a whole-body level neuronal, hormonal and mechanical/chemical receptors play a crucial role in regulating blood flow to all tissues.

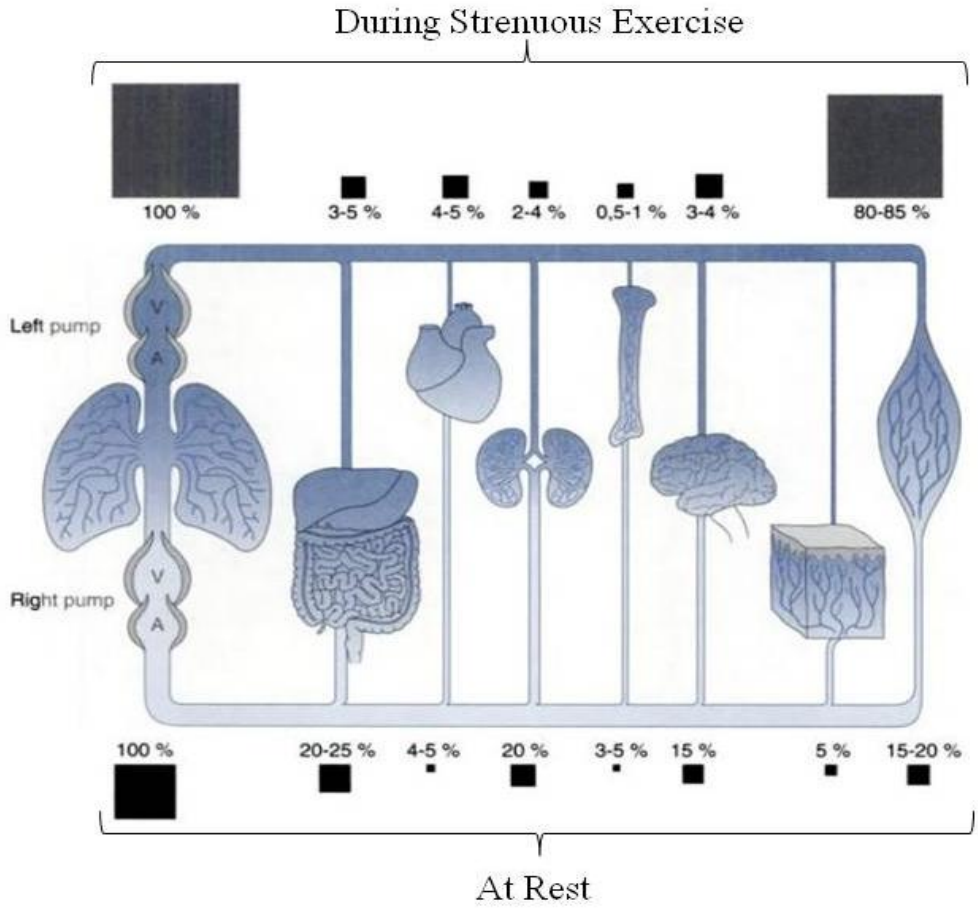

Figure 3. Relative distribution of blood to major organs at rest and during strenuous exercise.

The size of the black squares is roughly proportional to the minute volume of blood flow. (Not included is an estimated blood flow of 5% to 10% to fatty tissues at rest, about 1% during strenuous exercise) (Astrand, 2003).

However, arguably the most important factor is the vascular system itself. The vascular system (excluding the pump – heart) is basically made up of delivery (arteries, aterioles, to capillaries) and drainage (venules and veins) vessels with varying physical properties. The delivery vessels (although not the final vessels: capillaries) can alter their diameter to both maintain blood pressure and decrease/increase blood flow. The arteries have the largest diameter; with thick (relatively) walls that facilitate blood flow directly from the heart and can withstand high arterial pressures. The capillaries are the final vessels and and have a diameter barely wider than a single red blood cell (RBC) to enable diffusion of substrates into tissues, most importantly into (for muscle work) muscle.

Whilst all tissues/organs require an adequate blood flow, muscles influence on the CVS (due to its dynamic and energy needs) provides the biggest challenge, as the

greatest amount of blood vessels perfuse it. The average human body contains over 100,000 miles of blood vessels (McArdle *et al.*, 1996), of which it is estimated that capillaries constitute 60,000 miles (Junqueira & Carneiro, 2005). Repetitive and sustained muscle contraction, as in endurance exercise, significantly increases the local working muscles requirement for not only O_2, but for substrate delivery and, CO_2 and waste removal. The body systems must react and increase blood supply to the working musculature to enable continuation of exercise and to supply the substrates after exercise has been terminated to replenish depleted stores of lipids, CHO and amino acids (AA). With repeated exercise sessions the chronically altered gene expression will result in beneficial CVS adaptations, including significant increases in local muscle structures (capillary and mitochondrial density) and properties (vasodilatation) (Lindstedt & Conley, 2001; Atherton *et al.*, 2005; Laughlin & Roseguini, 2008). For example, at rest the heart pumps approximately 5 L of blood min^{-1}, during strenuous exercise this can rise to 40 L min^{-1} (Ekblom, 1968), this huge change requires an efficient and concerted interconnected physiological and molecular response. The vascular network/structure is of particular importance because small changes in diameter of the conducting vessels equal large changes in blood flow and maintenance of blood pressure, which are both vital, especially during physical activity. Blood pressure must remain adequate to supply the brain with O_2 and all other organs despite the decrease in supply, which has been re-directed to the working muscle (Figure 3).

At a local ultra-structural level there are several key factors that facilitate enhanced O_2 delivery, including an up-regulation of vasodilatory peptides (nitric oxide – NO, bradykinin – BK, endothelium-derived hyperpolarising factor – EDHF (Clifford & Hellsten, 2004; Luksha *et al.*, 2009)), anti-clotting factors (thin the blood thus facilitating delivery) and increasing the number of open capillaries (Rattigan *et al.*, 2007) to enhance the surface area for diffusion.

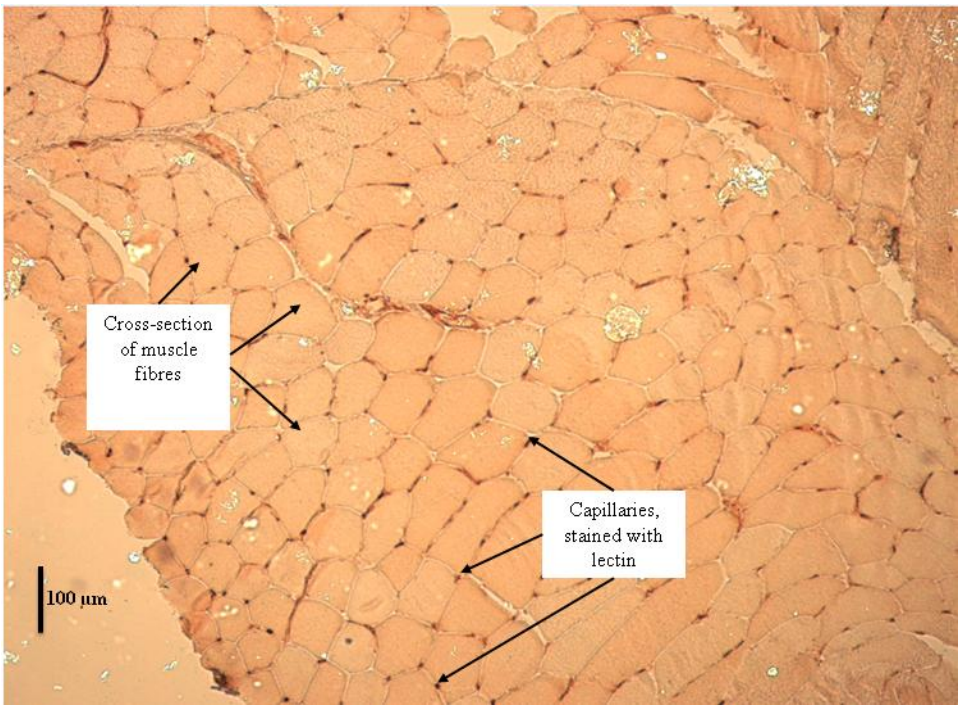

Figure 4. Cross-sectional area of human *vastus lateralis* muscle, stained for capillaries (lectin).

Taken from the one of the author's participants.

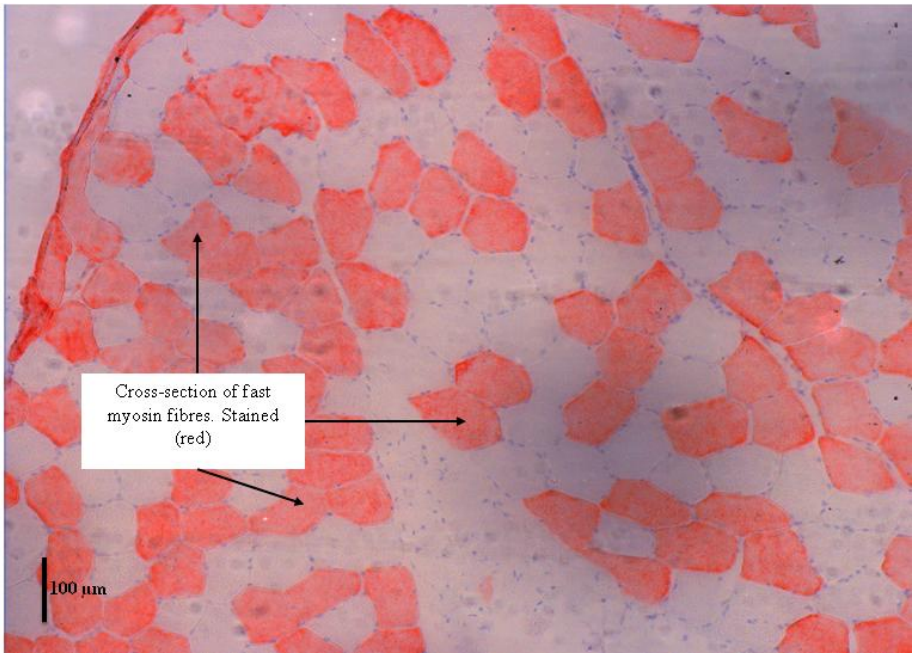

Figure 5. Cross-sectional area of human *vastus lateralis* muscle, stained for fast myosin (red).

Taken from the one of the author's participants.

Figures 4 and 5 show sections from muscle biopsies stained for both capillary density and fibre type. Human muscle is predominantly composed of a combination of slow (oxidative) or fast (glycolytic), with increased capillarity favouring oxidative – versus glycolytic - fibres (Laughlin & Roseguini, 2008) (Although, the actual muscle fibre itself does not ultimately dictate the capillary number or density (Egginton & Gaffney, 2010)). A study by Zoladz, et al. (2005) clearly showed (Figure 6) there was a difference, in capillary density, between untrained individuals and aerobically or strength trained individuals, but no difference between the two different trained groups (Zoladz *et al.*, 2005).

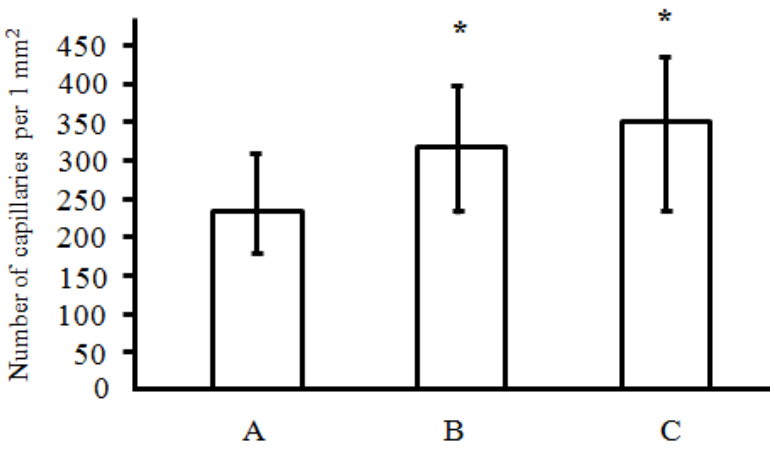

Figure 6. Capillary density in vastus lateralis muscle and training status.

(A): untrained students, (B): endurance athletes, (C): strength/power athletes. *significant difference between (A) and the two trained groups (B) and (C), but no difference between these two ($p<0.05$) (Zoladz, *et al.* 2005).

It is generally accepted that endurance training promotes a shift towards a slow muscle phenotype and increases capillary density – which will have significant athletic (increased capillary:fibre ratio and O_2 delivery) and health implications (Andersen & Henriksson, 1977; Hawley, 2002). For example, blood flow to supply muscle in exercising animals ranges from 60 – 400 ml/min for fast and slow fibre types, respectively (Hamilton & Booth, 2000), and a high percentage of fast fibre types is linked to metabolic diseases such as obesity and diabetes type 2 (DM2), (Hamilton & Booth, 2000) – see van Loon and Goodpaster, (2006) and Consitt, et. al., (2009) too.

Muscle can act like a metabolic sink utilising plasma glucose, free fatty acids, and triacylglyerides (TAG) – a very positive consequence of regular endurance type exercise. Yet if these metabolites are chronically elevated they can have a negative effect on human health due to excessive exposure. Low density lipoproteins (LDL) become oxidised and forms plaques in arterioles, which eventually leads to arteriosclerosis – a narrowing of the arteries.

Contracting muscle (specifically skeletal muscle) directly and positively impacts on many other organs: sustained skeletal muscle contraction such as during endurance type exercise results in increased heart rate. Over time the heart will hypertrophy, resulting in a decreased resting heart rate and reduced blood pressure (Mitchell & Victor, 1996). Studies have demonstrated that sympathetic tone is reduced after exercise training – contributing to decreased peripheral resistance and reduced hypertension (Serne *et al.*, 2006; Hansen *et al.*, 2010; Green *et al.*, 2011). Lipid metabolic pathways will also be prefentially utilised (something that elite endurance athletes benefit from through years of training), and glucose regulation (including a heightened sensitivity to insulin – resulting in less production) better controlled. Both of these two adaptations will result in reduced adipose storage (decresed risk of developing obesity) and decreased risk of developing DM2 – two of the most prevalent diseases of our modern lifestyle – both strongly linked and correlated with an excess of Calories and sedentariness.

There are clear beneficial adaptations resulting from regular endurance type exercise (also see Figures 9, 10, 14 and 15). Both visable to the eye (leaness, muscle definition, efficiency of movement), and clearly measureable at the physiological level (reduced heart rate and increased stroke output, greater oxygen uptake, increased muscle mass, increased capillary density, increased mitochondrial density, more compliant and reactive CVS, increase bone density – to name some). However, what role do specific genes or gene variants have on these adaptations in trained and untrained populations and even different ethnicities? This is a question this project aims to explore (see 1.2.7. Aims and Objectives of the research project).

1.1.2 Energy stores and supply of skeletal muscle

The two previous sections introduced both the importance of muscle and CVS that perfuses it. This section introduces the third main component that drives muscle work, energy substrates and their stores.

Endurance trained muscle is much more than just a force generating organ to enable movement: "skeletal muscle is an endocrine organ producing and releasing myokines (molecules released by contracting muscle: most notably cytokines and specifically interleukin-6 (IL-6); they can exert endocrine or paracrine effects; nitric oxide (NO) is another example) in response to contraction, which can influence metabolism in other tissues and organs" (Pedersen & Febbraio, 2008). Most importantly muscle makes a significant contribution to energy substrate storage and utilisation.

Muscle is not the only organ that requires energy, to contract to provide movement, in the form of ATP. Table 1 from (McClave & Snider, 2001) illustrates the various energy demands of organs in the human body at rest. Comparing absolute weights muscle appears to contribute little towards daily energy consumption. However, due to its sheer volume its role in energy expenditure is crucial. When you factor into the equation its ability to increase/decrease in size, quality and efficiency muscles importance to both energy expenditure and overall well-being heighten further.

Table 1. Energy requirements of various major human organs or tissues at rest.

Organ or Tissue	Metabolic Rate (kcal/kg/day)	Overall REE (%)	Weight (Kg)	Body Weight (%)
Adipose	4.5	4	15	21.4
Muscle	13	22	28.2	40
Other	12	16	23.2	33.1
Liver	200	21	1.8	2.6
Brain	240	22	1.4	2.0
Heart	400	9	0.3	0.5
Kidneys	400	8	0.3	0.5

Other refers to bone, skin, intestines and glands. Note: the lungs have not been measured for methodological reasons but have been estimated at 200 kcal/kg similar to the liver (McClave & Snider 2001).

There may be a limited amount of glucose that can be stored within the human body, but there is no limit to the amount lipids that can be stored (in fat cells – adipose tissue), which can be clearly witnessed by the burgeoning waistlines over the past three decades. Lipids can be stored subcutaneously (under the skin), viscerally (around organs) and within muscle (intramuscular triglycerides – IMTG), for the moment we will focus on IMTG stores. It was originally thought that high IMTG levels were associated with decreased insulin sensitivity, and thus increased risk of developing type 2 diabetes (DM2) and obesity (Consitt *et al.*, 2009; Muoio, 2010).

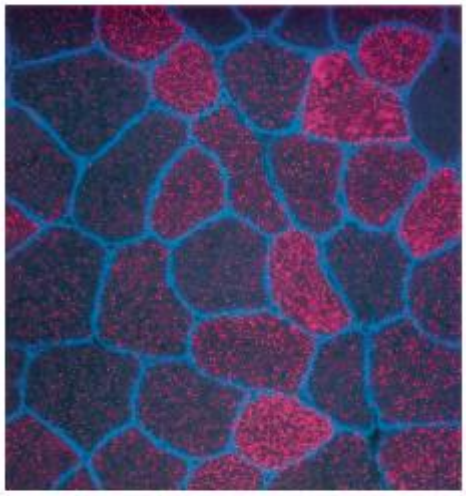

Figure 7. Stained section of rat tibialis anterior muscle.

Red fluorescent signal (Oil Red O) is the staining of intramyocellular lipids. The blue staining signal is the laminin of the cell membrane (Schrauwen-Hindering, *et al.*, 2006).

However, the 'athletes/metabolic paradox' where endurance training increases IMTG stores (van Loon & Goodpaster, 2006) and increases insulin sensitivity has somewhat clouded this line of thought. The IMTG droplets can be visualised in Figure 7 (Schrauwen-Hinderling *et al.*, 2006) and are often located adjacent (see Figure 8) to muscle mitochondria (van Loon & Goodpaster, 2006), potentially enhancing their availability to be utilised (van Loon & Goodpaster, 2006).

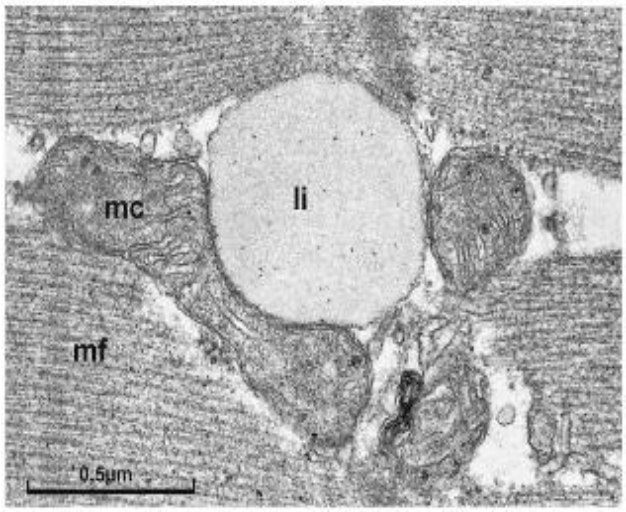

Figure 8. Electron micrograph of longitudinal section of muscle.
(courtesy of Hans Hoppeler)

li: lipid droplet, mc: central mitochondria, mf: myofilaments.

The increased use of lipids from IMTG stores for energy production (plus increased insulin sensitivity) in endurance trained people contrasts with obese and DM2 people (van Loon & Goodpaster, 2006). This suggests that it is not the amount of IMTG accumulated, but how it is accumulated (or turned over), which points to the very complex nature of physiological adaptations and the potential benefits of regular physical activity. Figure 9 illustrates this point, and Figure 10 provides further evidence of the importance of physical activity (especially aerobic type physical activity) and the relationship between the development of slow type fibre phenotype – and potential negative implications for health for humans with lower percentages (van Loon & Goodpaster, 2006; Consitt *et al.*, 2009).

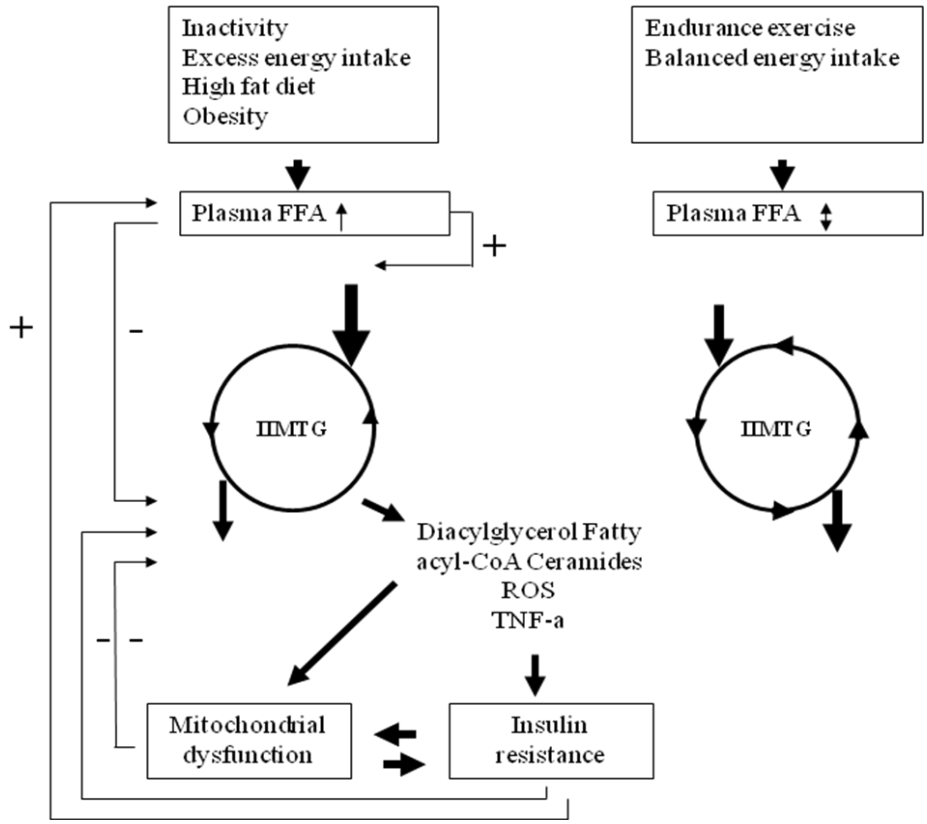

Figure 9. Contrasting metabolic pathways of inactivity and endurance exercise.

Inactivity coupled with excessive energy intake results in elevated IMTG storage, which produces a series of deleterious metabolic events possibly leading to DM2. Endurance exercise also elevates IMTG, but this is shown to be beneficial, allowing IMTG to act as a pool of nearby energy substrates, without the negative build up of potentially harmful metabolites (van Loon & Goodpaster, 2006).

Again we find that the mechanisms and amount of storage and usage, of glycogen, in physical activity endurance trained individuals is different (to sedentary). Endurance training (ET), providing CHO intake is sufficient, enhances glycogen storage, whilst sparing its utilisation by increased lipid oxidation (from white adipose tissue - WAT and IMTG). For example, if glycogen is the only energy source during relatively high endurance exercise (80% of VO_2max) it will be depleted in 60 – 90 minutes (Jones, 2004). Total (average) glycogen (liver + muscle), at an oxidation rate of 2.5 g/min would last for approximately 2 hours (at approximately 80% of VO_2max) (Frayn, 2003). Table 2 provides an example of the amount of energy substrates in a typical person.

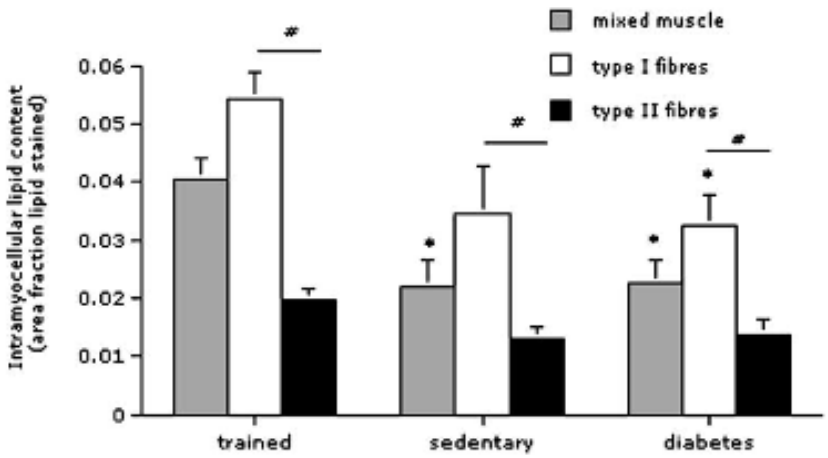

Figure 10. Fibre type and lipid content in humans: trained, sedentary and diabetic.

This simple figure illustrates the potential benefits of regular endurance training; 1) an enhanced percentage of slow fibre types and a concomitant increase in lipid stores. Note the similar fibre type and lipid content of both sedentary and diabetic individuals. Data provided are mean + SEM; *significantly lowered compared to values observed in trained athletes; #significant difference between type I and II muscle fibres (van Loon & Goodpaster, 2006).

The contrasting substrate utilisation processes and storage adaptations (as highlighted above) between different populations (trained, untrained (sedentary) and DM2) illustrates nicely how regular exercise can beneficially influence health. DM2 is a very modern disease and correlates strongly with increasing obesity (Williams & Pickup, 2004; WHO, 2005), of which both diseases correlate with sedentary type behaviour. DM2 individuals have a significantly greater risk of developing cardiovascular diseases (CVD) (Morrish, *et al.*, 2001; Huxley *et al.*, 2006). Figures (and related studies) 9 and 10 highlight local adaptations (in muscle) that occur with regular exercise (especially of aerobic type) and why these contribute to reductions in the risk of developing DM2 and the resulting CVD.

Table 2. Energy content of metabolic substrates (approximate values) of a typical 65kg person expending 10MJ/day.

Fuel	Amount (typical 65kg person)	Energy equivalent		Days supply if only energy source
		MJ	Kcal	
Carbohydrate				
Free glucose	12 g	0.2	48	0.02 (30 min)
Glycogen	450 g	7.65	1820	0.77 (18 hr)
Fat				
Triacyglycerol	15 kg	550	134,365	55
Protein	12.5 kg*	210	50,158	21

*not all body protein can be utilised (original reference Frayn, 2003)

The third major (potential) fuel, protein, can be broken down to release amino acids (AA), which can be oxidised during physical activity, even though its primary function is not energy provision: AA are crucial in providing structural and regulatory functions within the body (Tarnopolsky, 2004). Despite the intense marketing and media hype espousing the importance of large intakes of protein supplements (and individual amino acids) if energy requirements are met (from a mixed diet) then protein requirements will be adequately met – even in elite endurance athletes (Maughan, 2002; Tarnopolsky, 2004). Regular physical activity (especially of an aerobic nature) will result in increased amounts/activity of enzymes (related to energy metabolism, especially lipid related: for example; lipoprotein lipase (LPL), fatty acid binding proteins (FABP), carnitine-palmitoyl transferase-1 and -2 (CPT-1,-2)). Also, increased capillaries, haemoglobin, and myoglobin (as a result of regular exercise) – could in theory increase amino acid requirements (Tarnopolsky, 2004).

Human skeletal muscle can oxidise eight amino acids (Smith & Rennie, 1996), but the branch chain amino acids (BCAA) are preferentially oxidised during exercise (Lamont *et al.*, 1999; McKenzie *et al.*, 2000). Despite the obvious requirement of protein ingestion and turnover, its importance in relation to energy provision is likely minimal (providing carbohydrate intake is sufficient). It is estimated that oxidation of AA contributes between 1 – 6% of total energy provision – during endurance exercise (Tarnopolsky, 2004), although some estimates range up to 20% (Rennie *et al.*, 2006).

The carbon skeletons of AA (transaminated by enzymes within muscle cells) directly participate in ATP synthesis (via glycogenic or ketogenic pathways) (McArdle *et al.*, 1996). As the physiological functions of fibre types (in relation to type I and type II fibres – ignoring intermediate fibre types, which are likely of a small percentage) are different could the amount and utilisation vary during and recovering from exercise? In a resistance exercise study by Blomstrand and Essen-Gustavsson there were significant changes in levels of alanine, tryptophan, isoleucine and leucine; and glutamate, serine, alanine, tyrosine, methionine, valine, phenylalanine, isoleucine and leucine in plasma concentration during exercise and recovery respectively (Blomstrand & Essen-Gustavsson, 2009). What is most interesting from this study is the difference in concentrations or utilisation of AA between type I and type II fibres. The reduction in type II fibres, of glutamate, was twice that of type I fibres (70% versus 32%), suggesting the former make a greater contribution to force production

(Blomstrand & Essen-Gustavsson, 2009), which is generally well evidenced (Astrand, 2003; Jones, 2004). The concentrations of glutamate (decreased), and the BCAA (increased) were significantly different in type II versus type I fibres, immediately following a bout of resistance exercise (Blomstrand & Essen-Gustavsson, 2009). However, in an earlier study, (2002) by Blomstrand and Essen-Gustavsson, on the concentrations of AA, there was no significant difference in reduction of any AA (between type I and II fibres) following endurance exercise (cycling) (Essen-Gustavsson & Blomstrand, 2002). Although, aspartate, glutamate and arginine levels were all significantly lower, at rest, in type I fibres compared to type II. What was interesting in this study was, all the participants were endurance trained cyclist, and the day before they participated in glycogen depleting exercise (and did not eat until after the exercise test the following day). Therefore, did this muscle glycogen depletion influence AA usage, which resulted in an equal (relative) reduction in both fibre types? The authors seemed to think so. This depletion in AA would certainly make physiological sense, as AA (especially the BCAA) can be broken down (within muscle) and their carbon skeleton by-products utilised directly to produce glucose in gluconeogenesis (McArdle, 2005). (NB: in Blomstrand and Essen-Gustavsson fibre types were classified and pooled into groups of type I and type II – then the pooled types were weighed and then analysed for free AA).

1.1.3 Skeletal muscle phenotype and the influence of physical activity

The CVS and associated vessels (most importantly capillaries – where diffusion takes place) are responsive to external demands – by dilating/constricting and facilitating delivery and removal of substrates to muscle. Muscle itself is a very responsive/plastic organ. Muscle may comprise ~40% of a healthy males mass, but this is not fixed and will decrease with inactivity and increase with physical activity. Therefore muscle responds, or displays a plasticity to the environmental demands placed upon it (Figure 2). This plasticity involves an interaction between nature (genotype) and nurture (environment), which results in particular traits (e.g. increase force, fatigue resistance and underlying pathways such as a greater oxidative capacity etc) called a phenotype.

The contribution of nature versus nurture debate is hotly debated, with those leaning towards one or the other as the predominant influencing factor. In the author's opinion the influence of nature (genes) has been greatly exaggerated. Genes do not directly control any biological function or phenotype. Plus, when you factor in the number of genes which are up-/down-regulated by exercise, it becomes clearly apparent that to state any single gene or multitude of genes contributes a greater influence than environmental factors is overly simplistic. There is no gene for exercise – there is the conscious decision to perform the exercise and then there is the resultant adaptation. The striking differences in physiques of the two twins in Figure 2 illustrates the strong influence of environment.

A study by Janssen, et al. (2000) demonstrated increasing body mass is related to decreasing muscle mass (as a percentage of total body mass), which means increasing fat mass, equalling obesity (Janssen *et al.*, 2000), (Figure 11).

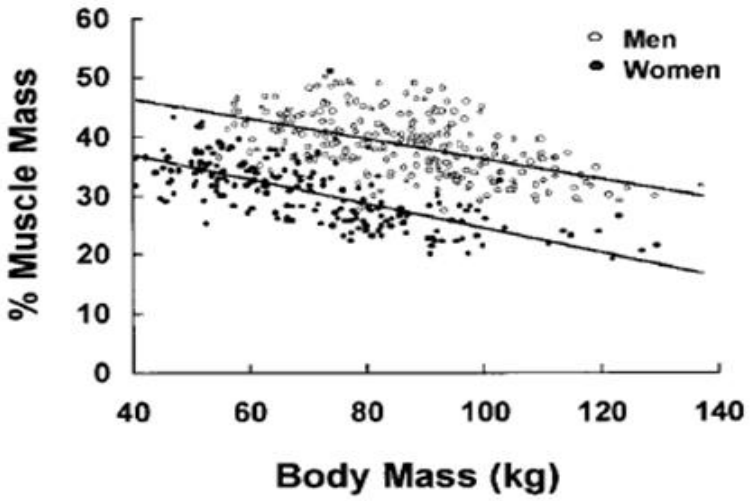

Figure 11. Relationship between relative muscle mass and body weight.

Solid lines, regression lines. Men: skeletal muscle mass = -0.208 (weight) + 43.4; SEE = 4.1. Women: skeletal muscle mass = -0.169 (weight) + 53.1; SEE = 4.3 (Janssen *et al.*, 2000).

In another study by Lee, et al. (1999) the benefits of being fit at different levels of body fatness and relative risk (RR) of dying from CVD are illustrated (Figure 12). The most pertinent point to take note of here is that it is better to be physically active (fit) and obese than lean and unfit – with relation to developing (and dying) from CVD.

These two simple illustration highlight the relationship (and importance) of regular physical activity and skeletal muscle clearly plays a key role.

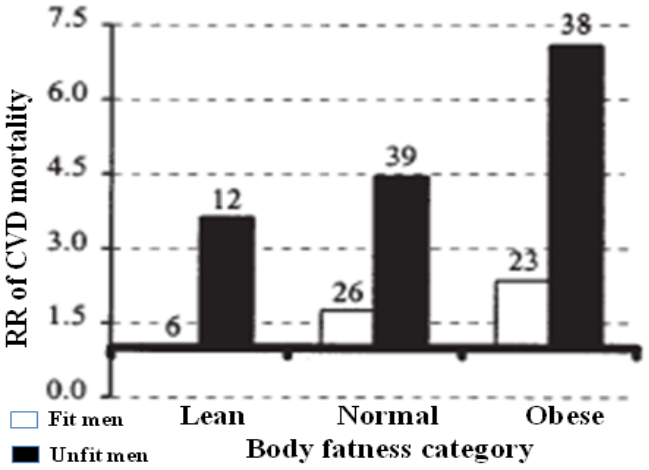

Figure 12. Body fat percentage and cardiovascular disease RR.

Fit lean men comprised the reference category, represented by the heavy line at 1.0. Unfit men were the lowest quartile of oxygen uptake ($mL^{\bullet}kg^{-1\bullet}min^{-1}$) in each age group, and fit refers to all other men. Body fatness categories were, in percentage body fat, lean (<16.7%), normal (16.7% to <25.0%), and obese (≥25.0%). Numbers above the bars represent the number of deaths (Lee *et al.*, 1999).

Research in the past decade highlights that the adjustments of the muscle phenotype, to a dominant stimulus like strenuous exercise, is specifically mediated by the activation of exercise-regulated gene expression signalling pathways (Fluck & Hoppeler, 2003; Booth & Neufer, 2005). This signalling response is specific for the type of exercise and induces pronounced adjustments in the levels of diffusible gene copies (transcripts/mRNA) in the recovery from the muscle stimulus. These significant adjustments in gene expression are translated via coupled protein adjustments, which then produce adaptations in contractile and metabolic function of muscle. Over time and with repetition (training) the exercise stimulus results in an accumulated adaptation of muscle strength and fitness. This is matched by the linear increase of biological variables from the level of single molecules up to muscle movement (Fluck, 2006).

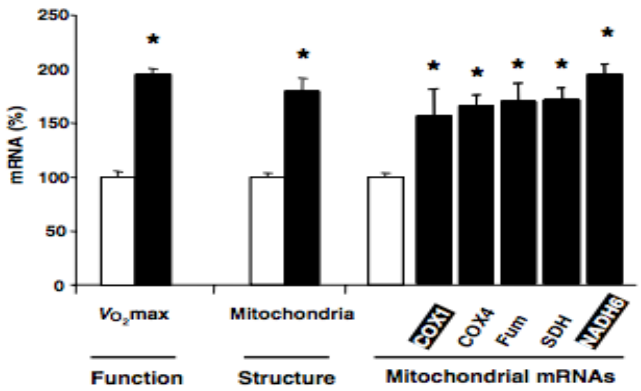

Figure 13. From the molecular to functional level: differences in trained and untrained participants.

■ Trained □ Untrained. Normalised VO_2max consumption, mitochondrial volume and mitochondrial RNA levels of various respiratory proteins in m. Vastus lateralis of endurance runners vs. untrained participants. mRNA levels are relative to 28S rRNA. Values are means ± s.e.m. Black boxes denote those mRNAs being encoded by mitochondrial DNA. Trained participants had been exposed to years of endurance training and competition. Significant differences between values from endurance-trained vs. untrained participants are indicated: *P<0.05. Enzyme list: COX1, cytochrome c oxidase subunit 1; COX4, cytochrome c oxidase subunit 4; FUM, fumerase; SDH, succinate dehydrogenase; NADH6, nicotinamide adenine dinucleotide hydrogenase (Fluck, 2006).

Figure 13 (from Fluck, 2006) illustrates the results of measures in trained versus an untrained population, analysing different systems (molecular: mRNA, ultra-structure: mitochondria, and function: VO_2max). There are clear statistical significant differences illustrated, but these results should come as no surprise.

The ability to utilise oxygen, at a whole-body level (a marker of cardiovascular fitness), will be related to processes at the molecular level. The blood vessels are essentially conduits for transporting metabolites (in this instance nutrients and oxygen) to the working muscle. Oxygen usage cannot simply be increased by delivering more oxygen to the working muscle – there needs to be metabolic processes in place that can utilise the nutrients and oxygen more efficiently. Simply put: there is an increase in the number of metabolite/energy producing biological "factories" – mitochondria (ultra-structure), which is mirrored by the increase in production of molecules (mRNA) which make up the mitochondrial organelle (to facilitate increased oxygen usage), and finally this is matched by a significantly higher usage of oxygen at the functional level (whole-body). Therefore individuals that partake in regular physical activity have a markedly different phenotype than sedentary individuals.

A detailed look into training-induced adaptations of muscle suggests that the

molecular response predicts how a given exercise stimulus instructs these adaptations (Schmutz *et al.*, 2006). A considerable inter-individual variability is observed to an initial bout of exercise, which is pronounced in the untrained state (Fluck, 2006; Schmutz *et al.*, 2006). A systematic analysis indicates that the activation of gene-mediated pathways primarily relates to the exercise stimulus and the prior history (phenotype) of the challenged muscle (Fluck & Hoppeler, 2003) Therefore genetic predisposition, phenotypic conditions (training state) and environmental influences (nutrition) are key factors that define this prior muscle phenotype (Pilegaard *et al.*, 2005; Fluck, 2006).

The manifestation of a particular set of related phenotypes such as an increase in the density of capillaries, mitochondria, and stroke volume of heart results in the development of an endurance phenotype. The complexity of the different systems and signalling pathways, plus environmental influences makes unravelling key components extremely challenging. Due to interactivity and possible redundancy in single bottlenecks a network approach is indicated (Oosterhof *et al.*, 2011), e.g. the possibility of discovering single components and/or single systems responsible for phenotypic changes is extremely remote when studying complex processes, especially those which evolve over many decades.

There are a number of related physiological changes resulting from being regularly physically inactive (Table 3) and physically active (Table 4). The heart (obviously a key organ in the CVS) undergoes beneficial adaptations resulting from regular endurance type exercise. Left ventricular hypertrophy and an associated increase in stroke volume coupled with a decrease in heart rate are just two adaptations that occur (in the heart) in an individual who regularly participates in endurance type physical activity (Gledhill *et al.*, 1994; Moore & Palmer, 1999). In muscle at the ultra-structural (or cellular) level, both capillary and mitochondrial density increase with regular endurance type physical activity (Gavin *et al.*, 2004; Schmutz *et al.*, 2006; Zoll *et al.*, 2006; Coffey & Hawley, 2007). These adaptations are necessary to meet the increased demand, on substrate supply via capillaries and mitochondrial metabolism required to fuel muscle contraction during sustained endurance physical activity.

Table 3. Unhealthy conditions precipitated by physical inactivity and resulting healthcare costs in the United States.

Unhealthy Condition	**Annual Cost of Condition (USD, $, Billions)**
Increased resting BP	286.5
Obesity	238
Type 2 diabetes	98
All cancers	107
Osteoporosis	6
Sacropenia	300
Back pain	28
Gallstone disease	5

There may be some overlap among some of the reported unhealthy conditions regarding cost, overt disease classification, and possibly with regard to biological mechanism. The main aim of the table is to illustrate the diversity of diseases that can develop with physical inactivity. The original references, for each unhealthy condition, can be found in the original article in Table 2 (Booth *et al.*, 2000).

Table 4. Beneficial effects of habitual physical activity.

Increase in maximal oxygen uptake and cardiac output – stroke volume
Reduced heart rate at given oxygen uptake
Reduced blood pressure
Reduced heart rate x blood pressure product
Improved efficiency of heart muscle
Improved myocardial vascularisation
Favourable trend in incidences of cardial morbidity and mortality
Increased activity of "aerobic" enzymes in skeletal muscle
Reduced lactate production at a given percentage of maximal oxygen uptake
Enhanced ability to use free fatty acid as a substrate during exercise, is glycogen saving
Improved endurance during exercise
Increased metabolism; advantageous from a nutritional view point
Counteracts obesity
An increase in the HDL-LDL ratio
Improved structure and function of ligaments, tendons and joints
Increased muscular strength
Reduced perceived exertion at given work rate
Increased release of endorphins
Reduced platelet aggregation?
Counteracts osteoporosis
Can normalise glucose tolerance

Please see reference 32 from the original article (Astrand, 1992), which this table was adapted from.

The next section will introduce the complexity at the molecular level, and related physiological adaptations to regular physical activity.

1.1.4 Molecular (and physiological) changes to physical activity, and health impact

The previous section looked at the physiological and beneficial health changes that result from regular physical activity. Figure 13 illustrated how molecular changes (mRNA) resulted in altered local adaptations (structure: mitochondria number) and linked physiological differences (increased VO_2max) in trained versus untrained humans. This section will expand on important molecular responses to regular physical activity and how they may result in important health protective effects.

As already alluded too, the blood vessels of the CVS are inextricably linked with the health and well-being of an individual. A crucial constituent of the vascular system is the vessel wall, which is obviously in constant contact with the circulating blood. Blood vessels are made up of several layers (see Figure 14), each producing different factors, which will impact on the functioning of any part of the vascular system.

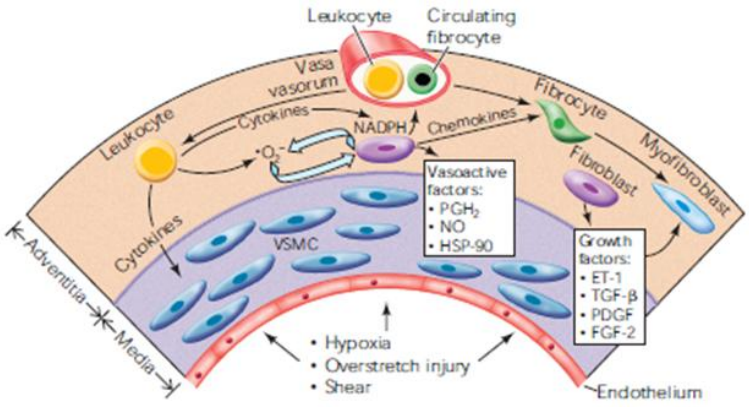

Figure 14. Basic structure of a blood vessel – cross-sectional

Abbreviations. NO: nitric oxide, PGH_2: prostaglandin H_2, HSP-90: heat shock protein 90, ET-1: endothelin, TGF-β: transforming growth factor beta, PDGF: platelet derived growth factor, FGF-2: basic fibroblast growth factor, NADPH: nicotinamide adenine dinuncleotide phosphate oxidase (Stenmark *et al.*, 2006).

We can see from Figure 14 that there are many molecular factors and events taking place within blood vessel walls. Shear stress (the viscous friction on the endothelial cell surface layer by blood flow: Jacobsen *et al.*, 2009), produced as the blood flows

over the endothelial cells, will alter these biological events, which will (over time) result in adaptations. Sustained laminar shear stress (LSS), which occurs in the linear/straight parts of the blood vessels (Pan, 2009; Traub & Berk, 1998) as produced by aerobic exercise has been shown (in vitro) to act as a stimulus for differentiation of human umbilical vein endothelial cells (HUVEC), which act as a protective/anti-atherosclerotic phenotype (Leung *et al.*, 2008). LSS is the "streamlined blood flow where viscous forces are predominant over inertial forces"; Pan, 2009. Therefore LSS blood flow is thought to be good, whilst oscillatory/low shear stress flow is thought to be bad and results in the development of lesions, located at branch points in the vasculature (Pan, 2009). A study by Wasserman *et al*., (2002) identified at least 107 genes as candidates for regulation by LSS (Wasserman *et al.*, 2002). Figure 15 illustrates a small number of endothelial genes regulated by LSS – as a result of exercise.

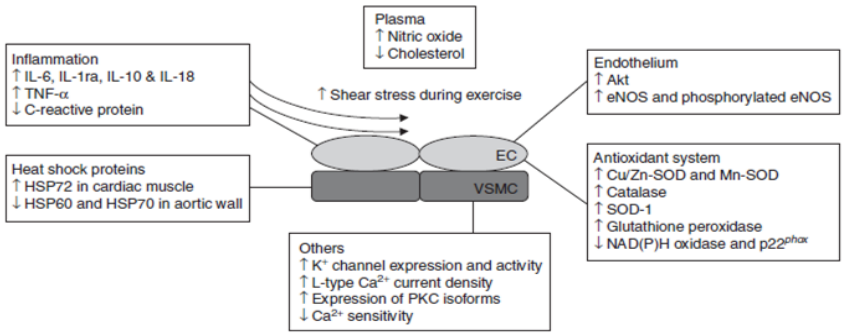

Figure 15. Direct effects of exercise on cells lining blood vessels

Endothelial Cells (EC) and Vascular Smooth Muscle Cells (VSMC).
See ref. Lueng, 2008 for specific details of genes in diagram (and full names) (Leung *et al.*, 2008).

Whilst the vast majority of people take up physical activity and cardiovascular type exercise to lose weight, the greatest benefits may result from non-traditional improvements (i.e. improved glucose/lipid metabolism, and a more plastic vascular system) that occur through regular aerobic type exercise – those that result in the development of a healthy endothelium and CVS (Thijssen *et al.*, 2010). Significant improvements in both conduit and resistance vessel endothelium function, in the absence of any improvements in traditional risk factors have been reported (Green *et al.*, 2003). Figure 16, again from Leung *et al.*, (2008) illustrates nicely the beneficial

effects of exercise on CVD risk factors including some proposed key molecular factors and processes thought to be involved/occurring and also highlights differences in arterio- and angio-genesis.

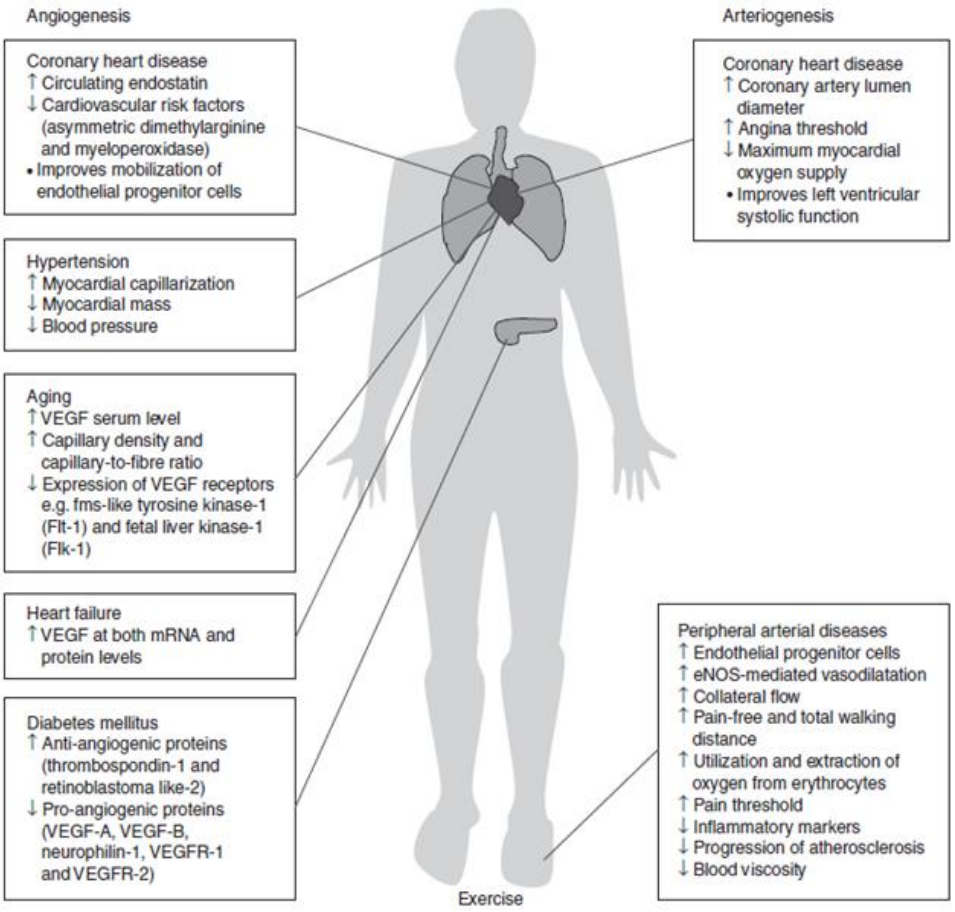

Figure 16. Benefits of exercise on vascular remodelling

See ref. Leung, 2008 for specific details of mentioned factors in diagram (and full names).

Most of the diseases of the CVS result from the malfunction of the blood vessel wall and vascular growth processes. There are three types of basic mechanisms of vascular growth: vasculogenesis (embryonic blood vessel formation), angiogenesis (sprouting or splitting of existing vascular capillary structures) and arteriogenesis (increase in diameter and wall thickness of arterial vessels) (Egginton, 2009; Persson & Buschmann, 2011). However, it is the latter two processes that are importantly altered by regular physical activity and in disease states. "A sustained increase of fluid shear stress (FSS) leads to activation of the endothelium" (Buschmann & Schaper, 1999). Whilst angiogenesis and arteriogenesis are two separate processes, initiated by

hypoxia and mechanical (stretch) and/or FSS respectively both share common factors like vascular endothelial growth factors (VEGF) and fibroblast growth factors (FGF) (Buschmann & Schaper, 1999). An example, of how angiogenesis and arteriogenesis can be affected differently by a disease state is illustrated in Figure 17.

Diabetes Mellitus (DM) is characterised by the malfunctioning control of blood glucose levels, by either destruction of the pancreatic cells that produce insulin (type I: DM1) or the desensitisation to insulin (type II: DM2) to allow glucose entry into cells for energy processes. The author proposes that defective neovascularisation signalling leads to increased angiogenesis and decreased arteriogenesis (Simons, 2005), which may be linked to the development of microvascular damage within the eye (retinopathy) and atherosclerosis (plaque development) – two diseases of the CVS that people with diabetes have an increased risk of developing.

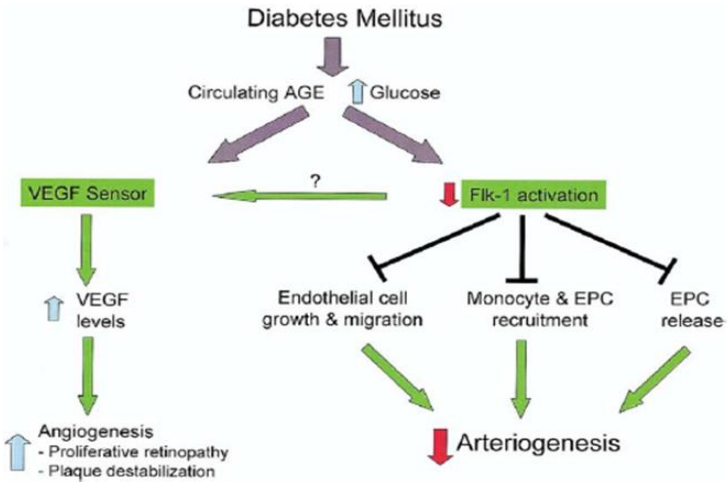

Figure 17. Proposed paradigm of neovascularisation abnormalities in diabetes

AGE = advanced glycosylated end-products. Defective VEGF signalling results in impaired Flk-1 activation that affects a number of processes thought to be involved in arteriogenesis, including endothelial cell growth and migration, monocyte and endothelial progenitor cell (EPC) recruitment, and EPC release by bone marrow. As a result, arteriogenesis is impaired. At the same time, decreased VEGF sensing, due to impaired Flk-1 activation results in increased serum VEGF levels that lead to pathological angiogenesis (retina artheroma) (Simons, 2005).

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Simons (2005) provides an insight into how DM2 may contribute to deleterious effects at a cardiovascular level. At its heart, diabetes is a disease where glucose homeostasis malfunctions, resulting in increased risk of many cardiovascular related conditions (Williams & Pickup, 2004). DM2 is probably the most strongly evidenced and classic lifestyle disease (linked to obesity), one resulting from excessive energy intake

coupled with inactivity (Williams & Pickup, 2004). DM2 is a metabolic disease, and has been steadily increasing over the last couple of decades, and approximately 400 (in the UK) people each day are newly diagnosed with diabetes (85% are DM2) (Diabetes, 2010) with no signs of abating. Figure 18 illustrates some very interesting physiological responses in DM2 participants compared to those without DM2. These findings (Figure 18 – D) suggest that impaired vascular function and reduced femoral blood flow affect low intensity exercise in DM2 patients (Lalande *et al.*, 2008).

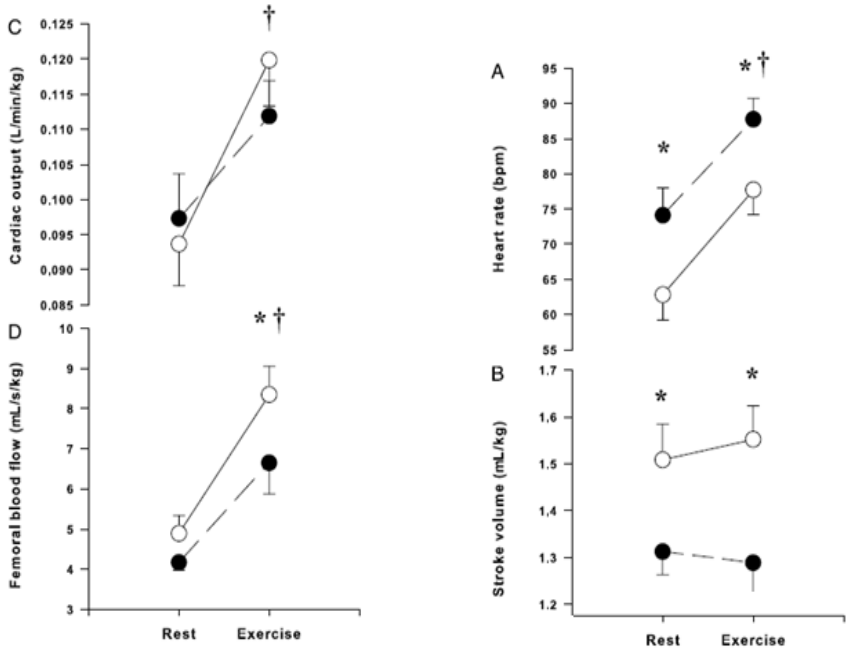

Figure 18. Responses to low-intensity exercise in patients with DM2

(A) Heart rate, (B) stroke volume indexed to fat-free mass, (C) cardiac output indexed to fat-free mass, and (D) responses to low-intensity exercise in patients with DM2 (black circles) and controls (white circles). * P<0.05 between controls and DM2 patients: †P<0.05 from rest to exercise in both groups (Lalande *et al.*, 2008).

Other studies have also demonstrated (in DM2 patients) a blunting of stroke volume (Baldi *et al.*, 2003), reduced cardiac output (Roy *et al.*, 1989), and impaired diastolic function (Baldi *et al.*, 2006). An obvious dysfunction of DM2 is raised blood glucose levels and studies have observed reduced blood flow is related to plasma blood glucose levels in patients with DM2 (Kingwell *et al.*, 2003).

Glucose is not the only energy substrate, when its metabolism malfunctions, that can result in a diseased state. LDL, which are important transporters of fat from

the blood to cells, are also strongly associated with a narrowing of arteries with increasing circulatory level and upon oxidation (oxLDL) (Hockley, 2007; Packard & Libby, 2008). However, as LDL is composed of different sub fractions, it may be the relative proportion of these and not the total amount in circulation that is important (Halle *et al.*, 1997). It is also possible that regular training, apart from favourably altering the sub-fraction content of LDL (Halle *et al.*, 1997), beneficially alters how LDL is metabolised. Figure 19 illustrates a significant reduction in femoral mean dilation (FMD) in sedentary older men versus exercised older men and the correlation to LDL-C levels (Walker *et al.*, 2009).

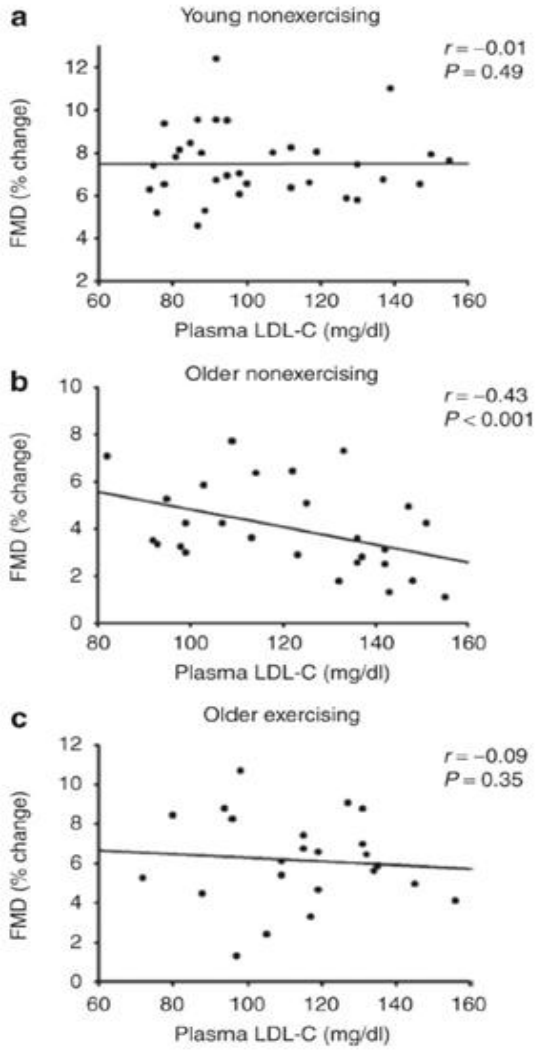

Figure 19. LDL-C and FMD relationship between sedentary versus exercising older men

Relationships between plasma LDL-C (low density lipoprotein-cholesterol) concentrations and brachial FMD (femoral mean dilation) in (a) young exercising men, (b) older non-exercising men, and (c) older exercising men. For each panel, men with optimal/near optimal and borderline high plasma LDL-C concentrations are pooled (Walker *et al.*, 2009).

Much like the contrasting development of laying down of IMTG stores within muscle (between trained healthy individuals and DM2 people – Figure 9) angiogenesis is equally complex. And it is how new blood vessels are formed and altered that is vitally important – not simply an up-regulation (of genes) or increase in density (capillaries) or change in vessel wall thickness.

"The first bout of exercise, after a period of rest, increases blood flow past endothelial cells (EC) in vessels, which, in turn, increases endothelial cell NOS (nitric oxide synthase - eNOS) protein activity, ultimately increasing its product NO" (Booth *et al.*, 2002). Release of NO may be the crucial factor that initiates the angiogenic pathway (Spurway & Wackerhage, 2006).

There is much evidence to suggest that exercise up-regulates NO (Hudlicka *et al.*, 2000; Silveira *et al.*, 2003; Prior *et al.*, 2004; Leung *et al.*, 2008; Woodman, 2008), and low levels/bioavailability of NO have been shown to correlate with increased risk of cardiovascular diseases (Naseem, 2005; Kocaman, 2009; Napoli & Ignarro, 2009). NO (and eNOS, which produces NO from endothelial cells) is an important molecule with potent vasodilatory actions, anti-atherogenic properties (Zhou *et al.*, 2001; Endres *et al.*, 2003; Kojda & Hambrecht, 2005; Naseem, 2005; Rush & Aultman, 2008; Napoli & Ignarro, 2009; Yung *et al.*, 2009), involved in important antiapoptotic and antiproliferative signalling pathways (Shyy & Chien, 2002) and antioxidant stimulatory functions (Kojda & Hambrecht, 2005). Consequently, NO is a marker of endothelial and vascular health (Kojda & Hambrecht, 2005; Napoli & Ignarro, 2009). Arterial stiffness (prognostic indicator in vascular diseases – cardiovascular arterial disease: CAD, DM2) amongst other factors is related to reduced endothelial production of NO (Woodman, 2008). NO also opposes the action, at the level of vascular smooth muscle cells (VSMC), to the bodys most potent vasoconstrictor, angiotensin 2 (Ang2) (Brillante *et al.*, 2008).

The evidence for the beneficial effects of regular physical activity is overwhelming, but the fine details explaining these benefits are sorely missing. Uncovering how molecular mechanisms work, in response to regular physical activity, will provide essential evidence to justifiably prescribe exercise/physical activity – just like a doctor currently prescribes medicine.

Cytochrome c oxidase (CYC1) may be amongst one of the most important antioxidant enzyme. CYC1 secures electron flow in electron transport chain (ETC)

thus decreasing potential superoxide (O_2-) formation. And the more mitochondria present (up-regulated by regular endurance type physical activity) equals more CYC1, which demonstrates an important and potentially beneficial molecular/ultra-structural adaptation to regular endurance type exercise. However, exercise does increase free radical production, which if not buffered can exert toxic effects (Ji, 2002; Banerjee *et al.*, 2003). A study by Zoll, *et al.*, (2006) demonstrated a significant increase in gene transcripts encoding factors of the ETC and antioxidant enzymes – following six weeks of endurance training (Figure 20) (Zoll *et al.*, 2006).

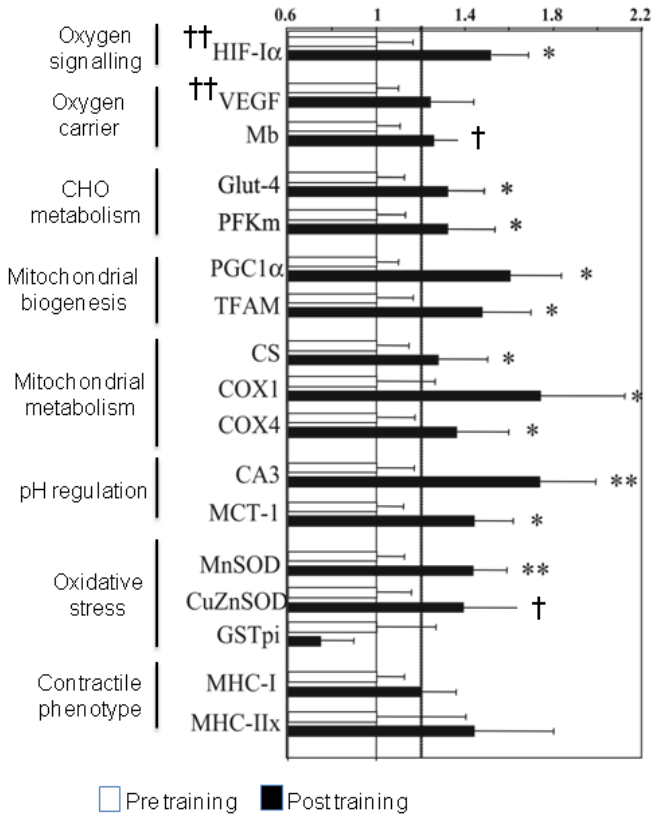

Figure 20. Gene expression changes after endurance training (in hypoxia)

**p<0.01, *p<0.05, † p<0.10, †† NO directly induces up-regulation of both these factors. (See Zoll, 2006 for an explanation of each of the abbreviated genes)

Although it is well established that many genes are differently expressed following endurance type exercise (Schmutz *et al.*, 2006; Klossner *et al.*, 2007), it was thought that these [genes] and regulatory genes returned to baseline levels if exercise was discontinued (Pilegaard *et al.*, 2000; Wittwer *et al.*, 2004). However Figure 21 (Wittwer *et al.*, 2004) illustrates the biological functions of proteins encoded by

mRNA's found to be different between the *Vastus lateralis* muscles of professional cyclists with many years of training and untrained subjects. The authors state "this indicates that permanent transcriptional changes (of regulatory genes) could contribute to the phenotypical differences of human muscle in a highly trained endurance-trained steady state" (Wittwer *et al.*, 2004).

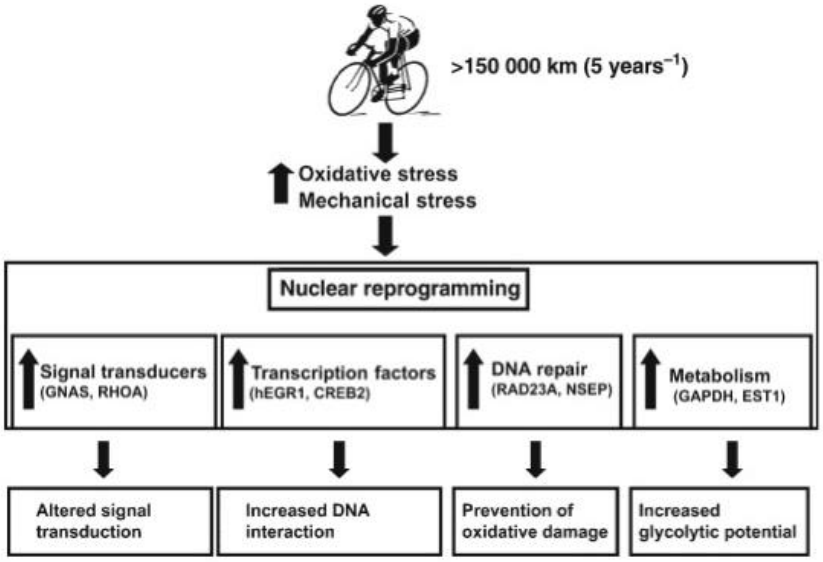

Figure 21. Important differences in proteins with many years of training

Summary of the biological functions of proteins encoded by the mRNAs found to be different between the *vastus lateralis* muscles of professional cyclists with many years of training compared to untrained participants (Wittwer *et al.*, 2004).

Therefore, an individual that regularly performs endurance type physical activity will likely have a very different expression of genes which relate to the altered physiological function and resulting phenotype(s). This phenotype will invariably be one that favours decreased risk of developing chronic diseases relating to the cardiovascular system. Booth and Neufer (2005) conclude that humans have developed a body that is primed for regular physical activity. Bramble (2004) also theorises, with good evidence, that humans have evolved over tens of thousands of years to be very efficient (and truly unique) endurance runners – with specific physiological adaptations rarely seen in other animals, such as achilles tendon, large gluteus maximus muscles, weight of head towards back (aids stabilisation), ability to sweat (Bramble & Lieberman, 2004).

As already alluded to, physical activity affects many system levels in the human body, which produces many beneficial changes. Phenotypes do not result from simple linear genetic interactions. Genes interact with other genes, which result in increased expression and stabilisation of mRNA to provide protein expression, which then results in the production of an end product – and over time physiological adaptations. Measuring gene expression, in isolation, can be misleading as increased gene expression does not necessarily correlate with increased translation, protein expression and altered function (Mata *et al.*, 2005). A relatively new field of study is metabolomics – "The metabolome is the quantitative complement of all low-molecular weight molecules present in cells in a particular physiological or developmental state" (Hollywood *et al.*, 2006). Recent studies have already been analysing the metabolomic response to exercise in blood plasma (Yan *et al.*, 2009; Lehmann *et al.*, 2010; Lewis *et al.*, 2010; Oberbach *et al.*, 2011). Metabolomics is attractive (much as transcriptomics first was when microarrays were developed) as it allows the simultaneous study of 100's (if not 1000's) of metabolites. Studying metabolite levels is also important because these are the end-products resulting from interactions between genes and the environment (Nicholson, 2006). These end-product mebabolites can then be further studied – in combination and in vitro – which is exactly what Lewis *et al.*, (2010) did. They found several metabolites that were significantly altered in response to endurance exercise – plasma markers of glycolysis, AA catabolism, and lipid metabolism. Some of these metabolites (niacinamide, glycerol, glucose-6-phosphate, pantothenate, and succinate) were then added to cultured myotubes, which resulted in a significant increase in the nur77 pathway (Burke *et al.*, 2010; Lewis *et al.*, 2010). Nur77 has been shown to be induced after exercise in muscle (Chao *et al.*, 2007), plus is a transcriptional regulator of glucose and lipid metabolism genes, in muscle (Chao *et al.*, 2007).

The biggest focus (as with much research) driving metabolomics is the hope of new discoveries of biomarkers for various diseases, which could eventually serve as targets for new pharmacological therapies (Holmes *et al.*, 2008). However, as exercise induces many beneficial pathways related to health and disease, studying the metabolomic response to exercise may lead to more specific exercise recommendations and some have even suggested the production of medicines that mimic the beneficial effects of exercise – "gymnominmetics" (Burke *et al.*, 2010). A

good example of the potential importance of using a metabolomic approach is represented by Yan, et al. (2009), where – amongst other investigations – they looked into metabolite differences (relating to energy usage and metabolism) between rowers and controls at rest, and found many important significant increases in blood serum of the rowers (see table 5) (Yan *et al.*, 2009).

Table 5. Serum metabolites significantly elevated in rowers compared to controls.

Related Metabolic Pathway	Retention Index	Metabolites	Control Subjects	Rowers
Glucose metabolism	1187.3	Alanine	122.8 ± 12.8	359.3± 21.4[c]
	1192.7	Lactate	1467.5 ± 134.5	3406.2± 212.1[c]
	2056.3	β-D-Methylglucopyranoside	8.14e6 ± 2.46e6	4.69e6± 1.49.e6[c]
Oxidative stress	1563.6	Cysteine	312.5± 26.0	487.7± 30.5[c]
	1627.8	Glutamic acid	32.6± 3.7	51.0± 2.7[c]
Energy metabolism	1833.8	Citric acid	57.0 ± 12.8	40.3± 2.9[b]
Lipid metabolism	2037.1	Palmitic acid	260.4 ± 22.3	150.0± 11.9[c]
	2203.1	Linoleic acid	185.2 ± 21.7	83.0± 10.7[c]
	2208.1	Oleic acid	242.3 ± 27.6	93.5± 16.3[c]
Amino acids	1227.5	Valine	209.9 ± 44.8	467.7± 35.0[c]
	1781.0	Glutamine	369.3 ± 26.0	533.4± 43.7[b]
Unidentified	1193.7	CPU_DB5_1193.7_S[a]	1.61e6 ± 1.18e6	7.85e6± 1.95e6[c]
	1303.9	CPU_DB5_1303.9_S[a]	2.70e6 ± 7.73e5	4.24e6± 1.36e6[b]

Concentrations of metabolites (μM) are given as means ± SE of 12 control subjects and 16 rowers. [a]Peak areas normalised against internal standard. [b]Mean difference is significant at the 0.05 level; [c]mean difference is significant at the 0.01 level, compared with the control (MANOVA). CPU_DB5_RIx_S, unidentified compounds in serum samples with DB-5 capillary column with GC/TOF-MS, Lab of metabolomics, China Pharmaceutical University (CU; RI, retention index; x, retention time index value) (Yan *et al.*, 2009).

Studying the metabolite response, in concert with gene expression, ultra-structure adaptations and physiological changes, strengthens any resulting correlations and similarities resulting from a study intervention.

1.1.5 Genetic make-up influences disease risk and athletic performance

Whilst all humans obviously share a large overlap in genetic make-up there are discrete differences owing to gene polymorphisms and single nucleotide mutations that over time may influence metabolism (Pilegaard *et al.*, 2000; Tunstall *et al.*, 2002). Due to the density of blood vessels, energy processing and requirements, muscle's influence on whole-body metabolism is enormous. Whilst each individual can condition their own muscle, the scope of this varies considerably between individuals. And this is purely a result of genetic constitution (training stimulus being equal).

In 2005 the latest report on the Human Gene Map for Performance and Health-Related Fitness Phenotypes was produced (Rankinen *et al.*, 2006). The report highlighted 10 different phenotypes and 242 related loci, ranging from endurance to lipids, inflammation and hemostatic phenotypes all of which will have great potential to affect metabolic processes.

One of the reasons why elite athletes are such an interesting and suitable group of individuals to study is (mainly) because (researchers' reason) any "special" performance phenotype (trait) that they are displaying will have likely arisen as a consequence of their genetic make-up (e.g. specific gene or interaction of genes). However, slowly, other factors are emerging, which are diluting the importance given to genetic make-up in the development of sporting performance. "...no one is genetically designed into greatness and few are biologically restricted from attaining it" (Shenk, 2010). The study of transgenerational epigenetic inheritance is uncovering further evidence that it is the environmental exposures, which shape phenotypes and not genes (Morgan & Whitelaw, 2008; Whitelaw & Whitelaw, 2008; Arai *et al.*, 2009)

"To what extent are individual differences in performance determined by genetic factors, and to what degree by nongenetic factors?" (Klissouras, 1976). This statement when substituting health for performance is as equally pertinent as it was 35 years ago. Eating healthily and being physically active is no guarantee of good health, but the risks of developing chronic diseases are greatly reduced. Better healthcare and treatment including long-term medication have increased longevity, but at what cost? A life lacking in important nutrients and high in processed (high salt, refined sugars and saturated fat) foods, combined with chronic alcohol intake, cigarette smoking and stress will take its toll on an individual's health....eventually. Regardless of lifestyle

some people are more prone to develop certain diseases and/or have greater athletic ability. Therefore it is logical to presume some people must have protective/beneficial (and others deleterious/negative) mechanisms. And an individual's genetic make-up will significantly contribute to these mechanisms.

One simple method to analyse the affect of genes on performance is to study twins (identical versus non-identical). Maximum oxygen uptake (VO_2max) is classically used to assess aerobic (endurance) performance, with higher levels generally associated with greater sporting performance (Joyner & Coyle, 2008). VO_2max "....represents the integrative ability of – the heart to generate a high cardiac output, total body haemoglobin, high muscle blood flow and muscle oxygen extraction, and in some cases the ability of the lungs to oxygenate the blood" (Joyner & Coyle, 2008). Although the importance placed on O_2 uptake is often over emphasised, and that there are many other components (technique/efficiency, muscle composition, energy stores and energy pathways, cardiovascular system, and mental strength.....etc – see Joyner, 2008 for a good review), which play an equal role in endurance performance (Lindstedt & Conley, 2001; Joyner & Coyle, 2008). However, measuring maximum oxygen uptake (VO_2max) is a simple and reliable method for providing an indication of aerobic (endurance) performance.

There is a huge amount of variability of VO_2max, both at baseline between individuals and in response to training (Bouchard *et al.*, 1997; Timmons *et al.*, 2005). How important is genetics to both baseline and trainability of VO_2max? Figure 22 illustrates the intrapair differences of VO_2max between identical and non identical twins. The authors stated that the difference between monozygotic (MZ) and dizygotic (DZ) twins (relating to intrapair variance) was significant, $P<0.01$ (Klissouras, 1976).

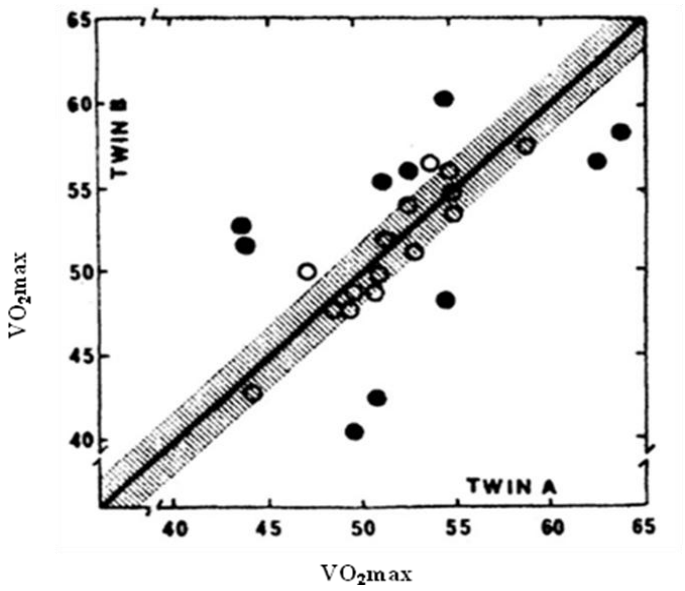

Figure 22. Intrapair values of maximal oxygen uptake for identical and non identical twins

Identical (○) and non identical (●) twins. This graph illustrates that VO_2max is largely determined by genetics. The intrapair VO_2max differences for the identical twins (depicted by open circles) is minimal – if the circles are on the line there is no difference. Whilst the intrapair differences for the non-identical twins (depicted by closed circles) is much more variable. In the original study the authors state that because the intrapair differences are so small (between the identical twins) these differences could be explained by experimental measurement errors (Klissouras & Marisi, 1976).

There is no sporting performance (to this author's knowledge) that is the result of a single gene – and it is unlikely there ever will be. "No genes can act in a vacuum, nor [can] phenotypes....develop and be actualised without the action of environmental forces", (Klissouras, 1997). However, evidence from epidemiology studies does point towards genes influencing a component of exercise-related phenotypes (Rankinen & Bouchard, 2008). Table 6 illustrates the main phenotypes (associated and investigated), papers published and number of different loci studied up to 2005 (Rankinen & Bouchard, 2008).

Table 6. Number of research articles and genetic loci summarised in the 2005 update of the Human Gene Map for Performance and Health-Related Phenotypes.*

Phenotype	# of papers	# of loci
Endurance	53	37
Strength and anaerobic	23	20
Hemodynamics	44	48
Body composition	37	34
Insulin and glucose	16	25
Lipids, inflammation, hemostatic	32	21
Chronic diseases	7	7
Exercise intolerance	52	31
Physical activity	6	14

*See reference 29 in original article (Rankinen & Bouchard 2008).

A study looking at endurance performance related polymorphisms identified 23 where at least one significant association study was published (Williams & Folland, 2008). The authors concluded that the possibility of one individual holding all 23 polymorphisms was 1:1212 trillion. The authors of this study also developed a total genotype score (0-100), which was used in another study looking at 14 endurance related polymorphisms – see table 7 (Ahmetov *et al.*, 2009).

In 2010 Rankinen (Rankinen *et al.*, 2010) reviewed only the very highest quality studies (in accordance with their study criteria) published (between 2008 and 2009) on exercise, fitness, performance genomics and health. The genomic variations that they reviewed were all related to important fitness and/or health linked phenotypes (ACTN3 R577X gene polymorphism), endurance performance/cardiovascular phenotype (mtDNA haplogroups, K1F5B and ITGB1 genes), regulation of blood flow (NOS3 gene) obesity (FTO gene), DM/insulin-glucose metabolism (PPARG, PPARD, PPARGC1A, NDUFB6 and ENPP1 genes), and lipid metabolism (APOE gene). The authors concluded that there was not enough high quality evidence to demonstrate conclusively that any gene or variant was associated with a particular performance/health phenotype.

Table 7. Candidate genes for endurance performance: their full names, functions of gene products, associated phenotypes and interactions.

Gene	Function
ACE	Regulates circulatory hemeostasis through the synthesis of vasoconstrictor angiotensin II and the degradation of vasodilator kinins
AMPD1	Regulates muscle energy metabolism by catalysing the deamination of adenosine monphosphate to inosine monophosphate
HIF1A	Regulates the transcription of numerous genes in response to hypoxic stimuli. Genes responsive to HIF1 are involved in the processes of erythropoiesis, angiogenesis, and metabolism and include those encoding erythropoietin, VEGF PPARα and glycolytic enzymes
NFATC4	Regulates cardiac hypertrophy, glucose and lipid metabolism, expression of the skeletal myosin heavy chain genes; regulates expression of PPARG
PPARA	Regulates liver, heart and skeletal muscle lipid metabolism, glucose homeostasis, mitochondrial biogenesis, cardiac hypertrophy, expression of UCP2 and UCP3
PPARD	Regulates fatty acid β-oxidation, glucose utilisation, mitochondrial biogenesis, angiogenesis, muscle fibre type, expression of PPARGC1A, UCP2, UCP3 and VEGFA genes
PPARG	Plays a critical physiological role as a central transcriptional regulator of adipogenic and lipogenic programs, insulin sensitivity and glucose homeostasis
PPARGC1A	Regulates fatty acid oxidation, glucose utilisation, mitochondrial biogenesis, thermogenesis, angiogenesis, formation of muscle fibres; co-activates PPARα, PPARδ; regulates TFAM and VEGFA expression
PPARGC1B	Regulates fatty acid oxidation, mitochondrial biogenesis, formation of muscle fibres; co-activates PPARα and PPARγ
PPP3R1	Confers calcium sensitivity, dephosphorylates and activates NFATC4. Regulates skeletal muscle and heart metabolism/hypertrophy, expression of HIF1A, PPARA, PPARD, PPARGC1A
TFAM	Involved in mitochondrial transcription regulation, proliferation of mitochondria and mitochondrial biogenesis
UCP2	Uncouples oxidative phosphorylation from ATP synthesis; regulates lipid metabolism and energy expenditure
UCP3	Uncouples oxidative phosphorylation from ATP synthesis; regulates lipid metabolism and energy expenditure, transports fatty acid anions out of mitochondria
VEGF	Growth factor active in angiogenesis, vasculogenesis and endothelial cell growth; expression of VEGFA is regulated by HIF1 and calcineurin/NFAT signaling

Abbreviations: ACE, angiotensin I-converting enzyme; AMPD1, Adenosine monophosphate deaminase 1; HIF1A, Hypoxia-inducible factor 1, α-subunit; NFATC4, Nuclear factor activated T cells, cytoplasmic, calcineurin-dependent 4; PPARA, Peroxisome proliferator-activated receptor α; PPARD, Peroxisome proliferator-activated receptor δ; PPARG, Peroxisome proliferator-activated receptor γ; PPARGC1A, Peroxisome proliferator-activated receptor γ coactivator 1-α; PPARGC1B, Peroxisome proliferator-activated receptor γ coactivator 1-β; PPP3R1, Protein phosphatise 3, regulatory subunit B, alpha isosform (calcineurin subunit B); TFAM, Mitochondrial transcription factor A; UCP2, Uncoupling protein 2; UCP3, Uncoupling protein 3; VEGFA, Vascular-endothelial growth factor A. Please see the original reference (Ahmetov *et al.*, 2009)

The brief overview presented in this section highlights the importance of performing research that is designed to try and uncover mechanisms behind supposed genetic associations – such is the variability in study findings. Only by trying to uncover molecular mechanisms and linking them to different system levels will it be possible to gain an insight into the influence (if any) of genes or specific variants (or groups - haplotypes).

To further highlight the difficulties and controversies of studying a single gene or gene variant and health or sporting performance phenotype the next section will look solely at one well studied (and potentially important) gene variant.

1.2.1 Introduction to ACE, ACE I/Dp research and controversies

The angiotensin converting enzyme (ACE) is the key regulatory enzyme in the renin-angiotensin system (RAS). The classical actions of the RAS (Figure 23) involve control of fluid balance and blood pressure (BP), and therefore it plays an important role in cardiovascular homeostasis, health and disease (Dzau *et al.*, 2001; Hall, 2003; Heeneman *et al.*, 2007; Fyhrquist & Saijonmaa, 2008).

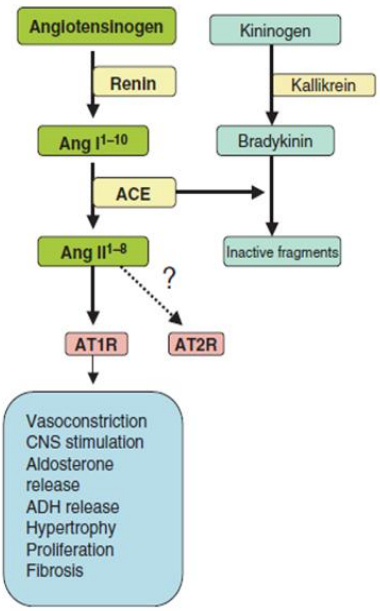

Figure 23. Simplified overview of the 'classic' circulating renin angiotensin system

Whole arrows indicate pathways of which the clinical significance has been demonstrated. Dashed arrow indicates pathway deduced from animal or cell culture experiments, not yet conclusively shown to be clinically relevant (Fyhrquist & Saijonmaa 2008).

Any scepticism regarding ACE's importance and role in CVD was firmly banished with the development of two effective medicines: the first being ACE inhibitors (ACEI) in 1977 (Ferreira, 2000), and then subsequently, angiotensin 1 receptor blockers (ATR1B) a few years later in the early 1980's (Skrbic & Igic, 2009). ACEI are now a primary medicine prescribed in the treatment of, not just high BP, but also for individuals with CVD (Dzau *et al.*, 2001). ACEI primarily act by reducing the catalytic activity of ACE thereby preventing the conversion of angiotensin 1 (Ang1) to angiotensin 2 (Ang2) (Figure 23). The latter, Ang2, is an important vasoconstrictory peptide of the vasculature. ACEI are believed to inhibit Ang2 production at both the systemic and tissue level depending on the pharmacology used (Dzau *et al.*, 2001). Therefore a frequent gene polymorphism that affected ACE activity levels may possibly correlate with outcomes in CVD risk. A study by Defoor, *et al.*, (2006) highlighted an improvement in aerobic power in ACE II genotypes (see below for description of genotypes) of patients with coronary artery disease (Defoor *et al.*, 2006).

In 1990 Rigat, *et al.*, (1990) discovered a 287bp *Alu* sequence in the ACE gene (Figure 24), classified when present as an insertion (I) or when absent (deletion: D), commonly termed the ACE I/D polypmorphism (ACE I/Dp) (Rigat *et al.*, 1990). This study demonstrated a linear relationship with the ACE I/Dp and an increase in serum/plasma ACE activity (ACE DD>ID>II). This observation was subsequently confirmed by many later studies (Butler *et al.*, 1999; Agerholm-Larsen *et al.*, 2000; Prasad *et al.*, 2000; Arcaro *et al.*, 2001).

Their discovery, together with the invention of real-time polymerase chain reaction (RT-PCR, (Deepak *et al.*, 2007)), produced a spurt of interest in the ACE I/Dp and disease (mainly CVD related). The search terms ACE Polymorphism + Hypertension or Infarct or Diabetes or Coronary have resulted in 1640, 1060, 685 and 228 research studies respectively (Gard, 2010) since Rigat's study in 1990. Other areas of research (not exhaustive) include dementia, depression, Parkinson's disease, and cancer (Gard, 2010), but the interest in these areas of disease is much less (the most research was on cancer studies identified, and these were only 58). Interestingly, the more recent article (Ceyhan *et al.*, 2011) reported a significant association with poor coronary collateral circulation in ACE I/Dp subjects without the I allele – and they hypothesised this may be because of endothelial dysfunction. The majority (far from

conclusive) of evidence points towards an increased risk of CVS diseases in carriers with the ACE D allele (see section 1.2.6).

In 1998, a new research area developed, (Montgomery *et al.*, 1998) investigating the influence of the ACE I/Dp and human performance. This article commented on the significant increase in frequency of the I allele in mountain climbers and (in a second study) an increase in duration of repetitive elbow flexion (following a physical training programme) – also in I allele carriers. Section 1.2.6 provides an overview of the ACE I/Dp and human performance research.

Even with all the controversies and disagreement in the literature, there does not appear to be a decreasing interest in the ACE I/Dp. Since 1996 (obviously a slight delay from Rigat, et al's study) 70 to 119 articles are identified, based on PubMed searches, have been published yearly on ACE I/Dp. To date this year (01/01/11 - 09/11/11) 86 articles have been published. Much of the highlighted controversy in the literature revolves around the effect of the ACE I/Dp and human performance. Ethnicity, underpowered studies and mixed sporting populations have all been cited as reasons (main) for disagreement in study findings (Woods, 2009).

ACE I/Dp controversies: The following sub-section does not intend to address every controversy or to explain the disparity in the literature. What it does intend to do is highlight why, when studying single polymorphisms like the ACE I/Dp and relating them to complex phenotypic traits, like those of the cardiovascular system or human performance, a different experimental approach is required to better understand physiological effects in whole-organisms, such as humans.

Due to the central role of ACE in the CVS and the importance of the CVS in human performance much of this section will concentrate on the literature surrounding the ACE I/Dp and studies of the CVS (plus human performance). However, to help better understand the complexity, a range of different studies on the possible interaction of the ACE I/Dp within an intact whole-organism will be covered. The main aim is to bring together studies looking at different system levels to build a picture of how the ACE I/Dp may be influencing phenotypic adaptations. An example of the different types of ACE/ACE I/Dp studies, are illustrated in Figures 24 - 32.

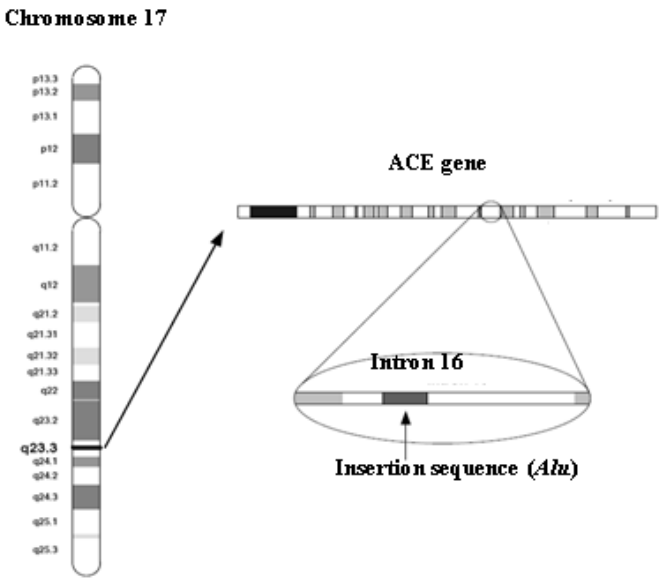

Figure 24. Location of the ACE I/D polymorphism

The presence (insertion) or absence (deletion) of a 287 bp sequence (*Alu* – see section 1.2.5. for more information) is what defines the ACE I/Dp, and this *Alu* sequence may affect the efficiency of transcription and/or activity of the resulting protein: "We believe the amplitude of the '*Alu* phenomenon' in both human genome and transcriptome has been only partially uncovered" (Hasler & Strub, 2006).

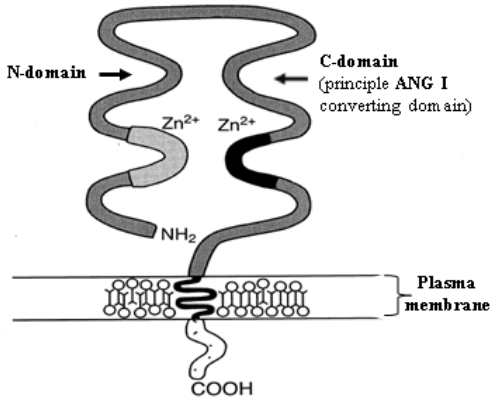

Figure 25. Different domains and effeciencies of the ACE protein

The ACE enzyme has two active sites (see section 1.2.2.), which are both capable of converting Ang1 to Ang2 (van Esch *et al.*, 2008). However, it has been demonstrated that the C-domain is the main Ang1 to Ang2 converting site, and is the only active domain in the D-allele (van Esch *et al.*, 2008) (see section 1.2.2. for more detail).

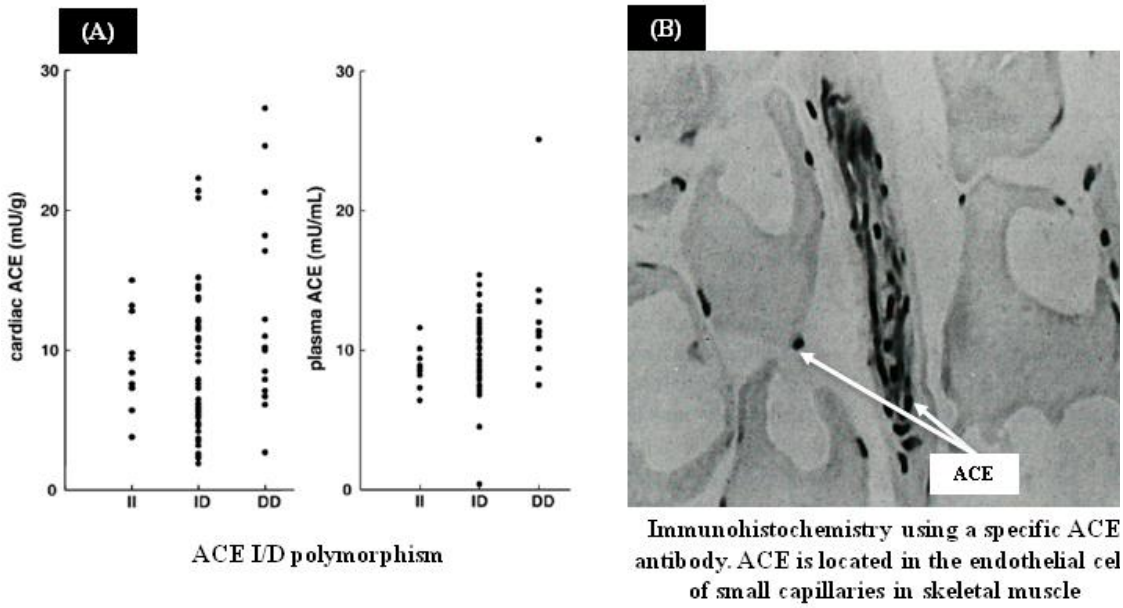

Figure 26. Levels of ACE (A) and location in skeletal muscle (B)

The authors of the original study (who discovered the ACE I/Dp – (Rigat *et al.*, 1990)) stated that 47% of the phenotypic variance of serum ACE was accounted for by ACE I/Dp. Although its (ACE I/Dp) influence may not be quite as high (Danser *et al.*, 2007). Figure 26 (A), relating to levels of ACE in cardiac – left figure – and plasma – right figure) come from studies, which state that the ACE I/Dp accounts for 22% and 31% respectively (Danser *et al.*, 1995; Osterop *et al.*, 1998). Research by Danser and group concentrate more on the pharmacological effects of ACEI, and may possibly ignore important contributions of environmental factors like sweating, exercise and stress – all of which would influence serum ACE and potentially Ang2 production and effects – thus phenotypic differences of serum ACE may well be as high (if not higher) than originally stated (Rigat *et al.*, 1990).

Figure 26 (B), illustrates where ACE is localised (which is where most active conversion occurs – (Danser *et al.*, 2007)) attached to endothelial cells lining capillaries (Schaufelberger *et al.*, 1998) as can be visualised here. In the same study an immunohistochemical staining of Ang2 was also illustrated, which showed a remarkably similar staining pattern – which is to be expexted as ACE produces Ang2.

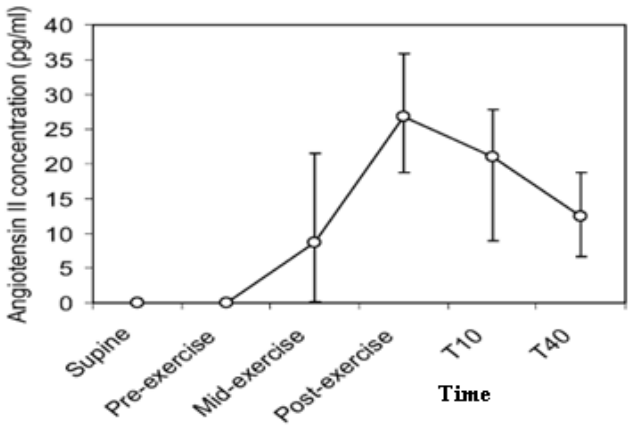

Figure 27. Ang2 levels at rest, during and post exercise

From Figure 27 we can clearly see that exercise increases Ang2 levels (Woods *et al.*, 2004) significantly – although there is little evidence to suggest ACE I/Dp influences this response. This is not that surprising for a number of reasons, some of which are: very low baseline circulating levels, very short half-life (30 seconds), and the actual techniques (from withdrawing blood, adding inhibitor chemicals to prevent degradation/potentiation, to actually running the assay and interpreting the results) required to accurately measure Ang2 (see section 1.2.3. for more information).

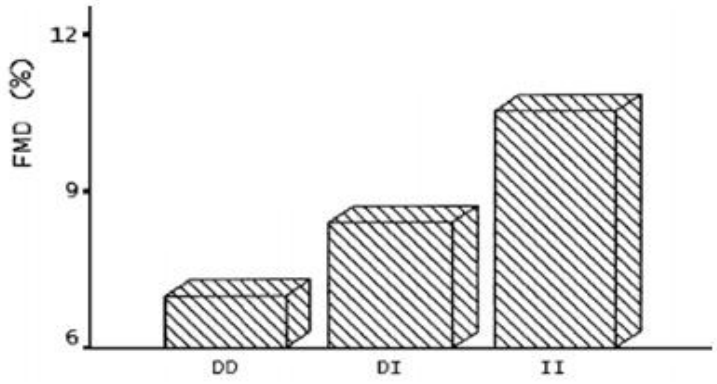

Figure 28. Varying vasodilatory response in artery diameter in ACE I/D polymorphism subjects

In Figure 28 Buikema, *et al.*, (1996) demonstrated a greater efficiency in converting Ang1 to Ang2 in ACE DD genotypes and methacholine-induced relaxation was also smaller in patients with the D allele. Figure 28 also illustrates the significant differences ($P<0.0001$) in flow-mediated dilation (FMD) in ACE II genotypes compared to both ACE ID and DD genotypes (Tanriverdi *et al.*, 2005). See section 1.2.6. for further details.

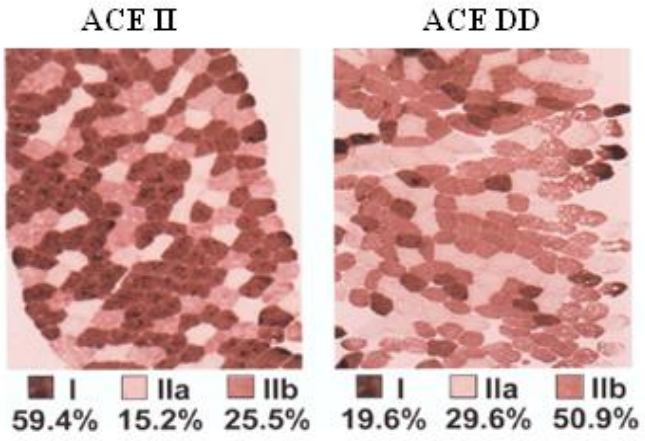

Figure 29. Sections of human skeletal muscle and percentage of fibre types in ACE II vs. ACE DD

Figure 29. Much of the research surrounding sporting performance and the ACE I/Dp proposes those individuals with the I allele have increased endurance capacity (see section 1.2.6. for much greater detail and references) although the evidence is far from clear cut. In Figure 30 it can be clearly seen that ACE II individuals (Japanese healthy untrained participants) had a significantly ($P<0.05$) greater percentage of type I muscle fibres than ACE DD individuals (Zhang *et al.*, 2003).

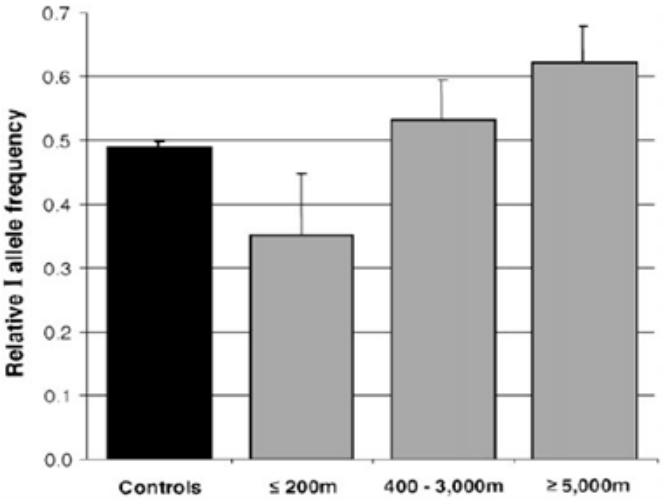

Figure 30. Relative I allele frequency of Olympic standard runners and controls

Figure 30 would appear to add to the theory that the ACE I allele is in some way related to an enhanced aerobic capacity. There is a significant ($P=0.009$ for linear

trend) difference in ACE I allele frequency with increasing running distance (Myerson *et al.*, 1999). Please see section 1.2.6. for further details.

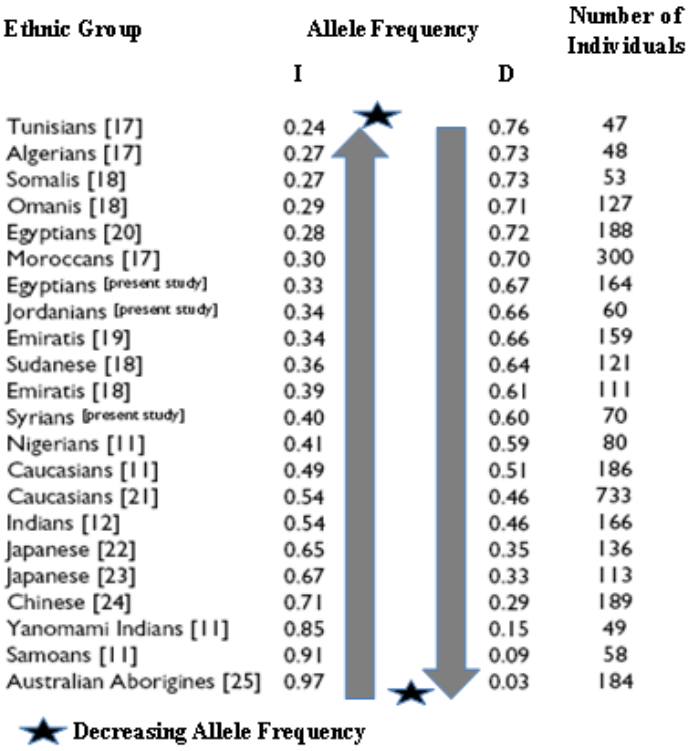

Figure 31. ACE I/D polymorphism allele frequency and ethnicity

Figure 31. It is well documented that certain ethnic populations are dominating long distance running events (see section 1.1.5.). What is also now recognised is the significant ACE I/Dp allelic variation between different ethnic groups – as highlighted here (Salem & Batzer, 2009).

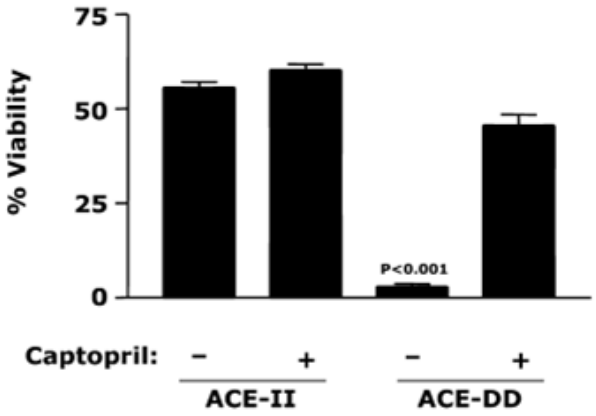

Figure 32. ACE I/D polymorphism and endothelial cell survival after slow starvation

Figure 32. The diversity of studies conducted on the ACE I/Dp really is quite remarkable. A study by Hamdi, et al. (2004) demonstrated, in vitro, human endothelial cells from ACE II participants had lower Ang2 levels and a 20-fold increased viability after slow starvation compared to ACE DD cells (Hamdi & Castellon, 2004).

Much of the early studies on the ACE I/Dp concentrated on single associations (endurance performance, blood pressure, coronary events), but as we can see in Figures 24 - 32 there are many factors to consider before making conclusions concerning the influence of a 287bp *Alu* sequence in a non-coding part of a gene. The ACE is a very important enzyme, with a key role in regulating vaso –constriction/-dilation. But there are many factors that interact to produce a particular phenotype, whether this be a specific disease or improved athletic performance. To truly understand what effect a specific variant within this enzyme can have it is imperative that as many linked-systems as possible are studied in concert. Figures 26 and 28-32 illustrates that any one study can suggest anything and another may support this theory. For example Figures 28, 29 and 30 all highlight significant differences in the I versus D allele: increased vasodilatory response, increased percentage of type I fibres, and increased performance in long distance running, respectively. These three studies all point towards increased capacity to deliver energy substrates and remove waste products (enhanced vasodilation) and better capacity to utilise these increased

substrates (increased oxidative fibres). This results in a superior performance over longer running distances.

This section has introduced how a single polymorphism in a key regulatory gene (of blood pressure) has been studied in relation to health and sporting performance. The next section goes back to the beginning of the discovery of the ACE gene and explains more about the RAS and its importance.

1.2.2 RAS and ACE: brief history and key discoveries

To truly understand a system (or anything) it helps to know its history. Table 8 below highlights the major discoveries of the RAS.

Table 8. Major discoveries of the renin angiotensin system (RAS).

Year	Discovery
1836	Bright (1836) discovered renal disease related to left ventricular hypertrophy (LVH) (Bright, 1836)
1872	High blood pressure measured for the first time (Mahomed, 1872)
1898	Renin discovered (Tigerstedt, 1898)
1940	Pressor substance discovered (later to be called angiotensin) (Braun-Menendez, 1940)
1956	Full RAS characterised (Skeggs *et al.*, 1956)
1965	Snake venom tested for potential inhibition of ACE (Erdos & Yang, 1967)
1972	Development of first ACEI begins (Byers & Wolfenden, 1972)
1977	First orally active ACEI synthesized (Ondetti *et al.*, 1977)
1982	Angtiotensin type I receptor blockers developed (Furukawa, 1982)
1988	Concept that RAS operates at the local tissue level as well as systemically (Dzau, 1988)
1990	Insertion sequence discovered in ACE gene related to decreased ACE activity (Rigat *et al.*, 1990)
2000	ACE2 (Donoghue, 2000)
2003	Orally active renin inhibitor first developed (Wood *et al.*, 2003)

Systemic versus local RAS: RAS was once thought of as purely an endocrine system with Ang2 conversion occurring in plasma and exerting its effects (vasoconstriction, sodium and water retention, and growth stimulation) upon binding to receptors (Abdel-Rahman *et al.*, 2004). More recently it is accepted that components of the RAS exist in virtually all cells (Muller & Luft, 1998; Lavoie & Sigmund, 2003; Haulica *et*

al., 2005; Fyhrquist & Saijonmaa, 2008) and has diverse actions (Watanabe *et al.*, 2005). Whether the entire set of components function locally is still a matter of debate (Danser, 2003; Fyhrquist & Saijonmaa, 2008). It is possible that one or more RAS components are taken up from the circulation (Abdel-Rahman *et al.*, 2004). Further, there is evidence of novel local effects of Ang2 including: proliferation, hypertrophy, protein synthesis (Li *et al.*, 1998; Gordon *et al.*, 2001; Jones & Woods, 2003), enhanced noradrenaline release (Saxena, 1992), ROS generation (Touyz & Schiffrin, 2000), increased energy expenditure (Cassis *et al.*, 2002), increases fatty acid and TAG synthesis (Jones *et al.*, 1997), to name just a few. Owing to a lack of understanding of the quantitative importance of local versus systemic effects and potentially novel functions, the role of the RAS in the development of many diseases (mainly CVD's) remains elusive. Although RAS (and its primary effector molecule Ang2) undoubtedly play important roles in development of many diseases especially those related to the CVS (Mehta & Griendling, 2007; Touyz, 2000; Heeneman *et al.*, 2007).

Related ACE molecules: ACE is not the only enzyme that can generate Ang2. Ang2 has been shown to be generated by chymase and cathepsin D via Ang1, and by elastase, cathepsin G and tissue plasminogen activator (t-PA) directly from angiotensinogen (AGN) (Belova, 2000; Danser, 2003; Rush & Aultman, 2008). Other angiotensin peptides can also be generated from Ang1 by ACE2 and other peptidases (Haulica *et al.*, 2005). It is reported that alternative Ang2 generation (other than by ACE) is roughly 40% (Rush & Aultman, 2008). However, the importance of alternative angiotensin generation is questionable. This is mainly due to the difficulties in accurate Ang2 measurement (see below), and differences between species (Danser, 2003). In addition *in vitro* studies may be providing misleading results. For example, a study by Kokkonen, *et al.,* (1997), clearly demonstrated that chymase Ang2 generation (in the heart) was strongly inhibited by proteases in the interstitial fluid (Kokkonen *et al.*, 1997). These inhibitory proteases would not be present *in vitro* and this would give misleading results, e.g. that chymase generates a significant proportion of Ang2, when in fact in a whole organism it doesn't (Zisman *et al.*, 1995; Kokkonen *et al.*, 1997). Evidence seems not to support the fact that Ang2 generation by any enzyme other than ACE is likely (*in vivo*) (Jorde *et al.*, 2000; Saris *et al.*, 2000; Campbell *et al.*, 2004;

Danser *et al.*, 2007). However, Table 9 lists some of the more studied possible ACE-Ang2 generating alternative enzymes.

Table 9. Alternative (potentially) ACE-Ang2 generating enzymes.

Component	**Significance**	**Site of origin and action**
- Chymase	Most likely an in vitro phenomenon, as located deep within the adventia and becomes inactivated if released into interstitial fluid (Danser *et al.*, 2007)	Chymases are mostly found in mast cells and extracellular fluid (Urata *et al.*, 1993; Wolny *et al.*, 1997). Detected throughout tissues, and in the adventia of vessel wall (Okunishi *et al.*, 1984)
- Cathepsin G	Possibly involved in local production of Ang2, modulating local blood flow due to inflammation (Dzau, 1989)	Chymotrypsin-like proteinase of neutrophils and spleen (Belova, 2000).
- Kallikrein	Probably not significant in vivo, as act (interestingly) kiningenase generating kinins at high pH (8-9) and Ang2 generating at low pH (4-6.5) (Maruta & Arakawa, 1983; Sasaguri *et al.*, 1997)	Produced as an inactive precursor by the liver (prekallikrein) (Corvol *et al.*, 1995)
- t-PA	In vivo can be secreted at sites of vessel damage / inflammation, and during hypoxia connected with restriction of normal blood flow (Belova, 2000)	Produced by the endothelium, and concentrated in blood vessel walls (Loscalzo & Braunwald, 1988)
Other angiotensins		
- Ang(1-7)	Interacts with NO and opposes actions of Ang2 (Watanabe *et al.*, 2005; Ferrario, 2006)	No specific origin or action. Generated by ACE2 from Ang2 (but can be generated from Ang1 or Ang2 by other peptidases. (Fyhrquist & Saijonmaa, 2008)
- Ang3	Generated from Ang2 by aminopeptidase A, and causes vasoconstriction (similar to Ang2). (Fyhrquist & Saijonmaa, 2008)	No specific origin or action. General vasoconstriction.
- Ang4	May have important regulatory functions in cognition, renal metabolism and cardiovascular damage (Savoia & Schiffrin, 2006; Ruiz-Ortega	May be generated from Ang3 by aminopeptidase M. (Fyhrquist & Saijonmaa, 2008)

	et al., 2007)	
Ang2 Receptors		
- AGTR-1	Mediates most of the known effects of Ang2 (Watanabe *et al.*, 2005).	Widely distrusted in all organs, basically the effector molecule of Ang2 (Mehta & Griendling, 2007)
- AGTR-2	Opposes actions of AGTR-1, upon binding Ang2 (Carey & Padia, 2008).	Mainly expressed in foetal tissue, and possibly during wound healing (Mehta, 2007}. Vasodilatory effects when activated.
- AGTR-4	Mediates the effects of Ang4 (Watanabe *et al.*, 2005).	Found in a range of tissues, including adrenal gland, kidney, lung and heart, and appreciable levels found in the brain. (Abdel-Rahman *et al.*, 2004)

Figure 25 illustrates the structure of ACE and that the majority (>90% - (Cushman & Cheung, 1971)) is found bound to endothelial cells of all blood vessels within tissues (Nash, 1992). Plus that conversion of Ang1 to Ang2 is mediated locally by tissue ACE (Maassenvandenbrink, 1998). Therefore it could be more beneficial to inhibit ACE at the local tissue level. This also may help to explain why patients with normal or low levels of systemic RAS activity can be effectively treated with ACEI (Brunner *et al.*, 1979; Dzau *et al.*, 2001).

Angiotensin Converting Enzyme Inhibitors (ACEI): In 2001-2002 the NHS spent just under one billion (£947,000,000) on anti-hypertensive prescription drugs (15% of total prescription costs) of which ACEI were the most costly contributing over one-quarter (£270,000,000) of the total costs (NEHGDG, 2004). ACEI are first line medicines for many CVD's and approximately £5.2 million is spent daily on cardiovascular medication in the UK (Bourn, 2007). The National Institute for Clinical Excellence (NICE) recommends that ACEI be indefinitely prescribed following myocardial infarction (NICE, 2007). It is clear to see that ACEI are ubiquitously prescribed, yet much needs to be understood about how they influence their many health benefits as, like virtually all drugs, they are not without substantial side-effects (see Table 10).

ACEI also potentiate the health benefits of BK as degradation will be slowed. In principle ACEI all have very similar actions (bind to and reduce ACE activity), but the potency and tissue retentiveness varies considerably. For example, quinaprilat has the greatest and captopril the least potency and tissue retentiveness (Dzau *et al.*, 2001). There are two active catalytic binding (although they may not have the same catalytic activity – but do produce the same product) sites on ACE (N- and C-domain – Nd, Cd –; amino- and carboxy-terminal respectively, (see Figure 28) (van Esch *et al.*, 2005), although the Cd is thought to be the main binding-site for conversion of Ang1 to Ang2 (van Esch *et al.*, 2008; Zhao & Xu, 2008). To add yet further complexity it has been demonstrated that only the Cd is active in the ACE DD genotype, whereas both domains are active in the ACE II genotype (van Esch *et al.*, 2008). In addition to these factors, ACE (Ang2 signalling cascade) is known to decrease with age (providing no related diseases that would significantly increase ACE-Ang2 signalling are present) (Cambien *et al.*, 1988; Dzau *et al.*, 2002), increased in atherosclerosis (Diet *et al.*, 1996), and its activity decreased potentially by certain plant bioactive compounds, as found in green and Rooibos tea (Persson *et al.*, 2010). All these factors highlight the complexity of the RAS (and this is only one small part of the RAS) and may also contribute towards much of the conflict between ACE related studies.

ACEI decreases Ang2 production which decreases – vasoconstriction, adhesion of monocytes, smooth muscle cell (SMC) growth/proliferation and migration, PAI-1 and thrombogenesis, matrix degradation, oxygen free radical production, endothelial dysfunction; ACEI potentiates BK action (reduced degradation) which increases – vasodilation, antiadhesion of monocytes, eNOS expression, PAI-1 and fibrinolysis, antiremodelling effect, antioxidant effect, preserved endothelial dysfunction (Bertrand, 2004; Ferrari, 2005). As with all drugs there are negative side-effects and ACEI are no exception (Table 10).

Table 10. Potentially negative side effects of ACEI.

Adverse Effect	Reference
Hypotension – usually due to taking along other hypertensive drugs and/or low sodium intake	(Brown & Vaughan, 1998a)
Hyperkalemia – due to decrease in aldosterone	(Warren & O'Connor, 1980)
Renal function impairment – decreased glomerular filtration (reversible)	(Kastner *et al.*, 1984)
Cough – mechanism unknown, possibly due to stimulation of vagal C fibres by increased levels of bradykinin or substance P	(Dicpinigaitis, 2006)
Angioedema – although quite rare (0.1-0.2% of prescribed patients)	(Israili & Hall, 1992)
Fetel abnormalities – although ACEI should not be prescribed is a women is trying to become pregnant and should be ceased immediately if they are pregnant	(Pryde *et al.*, 1993; Sedman *et al.*, 1995)
Neutropenia – very rare (<0.05% of patients)	(DiBianco, 1986)
Skin rash	(DiBianco, 1986)
Nephrotic syndrome	(DiBianco, 1986)

1.2.3 Angiotensin 2, receptors and signalling

Ang2 controls cell signalling via binding to seven-transmembrane receptors which activate G-protein dependent biochemical cascades (Touyz & Schiffrin, 2000). And the Ang2 type-1 receptor (AT1R) is thought to control most of the physiological actions of Ang2 (including Ang2 induced vascular functions) (Sadoshima, 1998; Mehta & Griendling, 2007). These are the classical physiological effects associated with blood pressure regulation such as vasoconstriction and aldosterone release and the regulation of growth and inflammation including general cell remodelling (Touyz & Schiffrin, 2000; Mehta & Griendling, 2007).

AT2R is another Ang2 receptor in humans. It is however mainly expressed during foetal development (Shanmugam & Sandberg, 1996) and with tissue regeneration during wound healing, in the heart and vascular system (Nakajima *et al.*, 1995), and binds Ang2 with similar affinity as the type 1 receptor (Jones & Woods, 2003). AT2R is thought to antagonise the action of AT1R through the stimulation of vasodilatation,

apoptosis, and anti-proliferative/-hypertrophy/-thrombotic (Fyhrquist & Saijonmaa, 2008).

Measurement of ACE and Ang2: To help understand the implication of the RAS in human disease and performance it is vitally important to accurately assess the activity of ACE and its main product the Ang2 peptide. Whilst ACE activity (plasma) has been shown to be very stable – blood samples can be safely left for up to 3 hours and levels are maintained (Jalil *et al.*, 1999), and are stable within individuals (Alhenc-Gelas *et al.*, 1991) the story is not the same for Ang2. There are consequently a number of biological and technical limitations concerning the measurement of ACE and Ang2, as described next. ACE activity is: 1) mainly at the tissue level (Dzau *et al.*, 2002), and measures typically ignore the fact that only 10% of total ACE activity is found circulating (Cushman & Cheung, 1971), 2) altered by the health of the endothelium, e.g. upregulated by atherosclerosis (Diet *et al.*, 1996; Dzau *et al.*, 2002), 3) influenced by genetics, e.g. ACEI/Dp, ethnicity (Payne *et al.*, 2007), and 4) decreases (and then stabilises) with age (Cambien *et al.*, 1988; Dzau *et al.*, 2002). Also, accurately quantifying Ang2 (circulating) is very challenging for a number of reasons: 1) its half-life is (in circulation) estimated to be 30 seconds (van Kats *et al.*, 1997), but with an intracellular half-life of 15 minutes (van Kats *et al.*, 1997), 2) generation and degradation of Ang2 *in vitro* as a consequence of freezing and thawing (Nussberger *et al.*, 1986; Abdel-Rahman *et al.*, 2004), 3) cross-reactivity of different angiotensins with Ang2 (Lijnen *et al.*, 1978; Nussberger *et al.*, 1986), and 4) venous sampling is not reflective of arterial delivered Ang2 (Lijnen *et al.*, 1978). These considerations suggest explanations for the inconsistency regarding the relationship between increased ACE activity (and ACE I/Dp) and Ang2 production/levels.

There is also the small, but very important matter of individual dietary differences, which affect the RAS, mainly sodium (Staessen *et al.*, 1987). Plus there are bioactive components in food that mimic ACEI's, which will again affect ACE levels (Actis-Goretta *et al.*, 2006). Finally, Ang2 levels are at extremely low concentrations in human plasma, estimated to be less than 1 – 149 pg/ml (Lijnen *et al.*, 1978; Nussberger *et al.*, 1986; Staessen *et al.*, 1987; Hespel *et al.*, 1988), which makes accurate assaying difficult, especially in samples with very low levels.

1.2.4 ACE, bradykinin and vasodilation

An intriguing fact of long-term treatment with ACEI is that this results in the elevation of ACE levels (Fyhrquist *et al.*, 1980). Yet, they [ACEI] still convey beneficial effects like decreased: Ang2, anti-inflammatory factors, free radical production and endothelial dysfunction, and increased: BK, vasodilation, antioxidants, antiadhesion of monocytes and preserved endothelial function (Bertrand, 2004; Ferrari, 2005). The possible reason for this may be due to the reduced inactivation of BK, which is a molecule with strong vasodilatory properties. ACE not only activates Ang2 it also inactivates BK, and ACEI reduce this – therefore potentiating BK's vasodilatory functions. This is an important observation: that ACE has a much greater affinity for BK than Ang1. Therefore, the benefits of ACEI may not be entirely due to a reduction in Ang2 and vasoconstriction, but also reside in the increase in BK-mediated vasodilation (Jaspard *et al.*, 1993). BK (and kinins) exerts more than vasodilatory effects, it has many known cardiovascular effects, some of which are: increased vascular permeability induction of hypotension (Bhoola *et al.*, 1992), induction of anti-inflammatory pathway (Brown & Vaughan, 1998a; Woods, 2009).

BK binds to one of two receptors, of which BK receptor 2 (BK2R) is constitutively expressed, whilst BK1R is expressed (normally) after tissue injury (Muscogiuri *et al.*, 2008). Upon binding to BKR2, BK activates the eNOS and this produces NO (Erusalimsky & Moncada, 2007; Muscogiuri *et al.*, 2008). The NO molecule is so small that it diffuses freely through cell membranes. It is produced by a variety of cells including (importantly) endothelial cells and human muscle fibres (Clifford & Hellsten, 2004). NO has also been implicated in many important biological functions (Filippin *et al.*, 2009). Its main effect is to induce a vasodilatory response in the vasculature (Clifford & Hellsten, 2004; Filippin *et al.*, 2009). Thus BK, through activation of NO, opposes the action of Ang2. Figure 33 illustrates some of the key signalling interactions induced by BK2R activation by BK and its activation of NO.

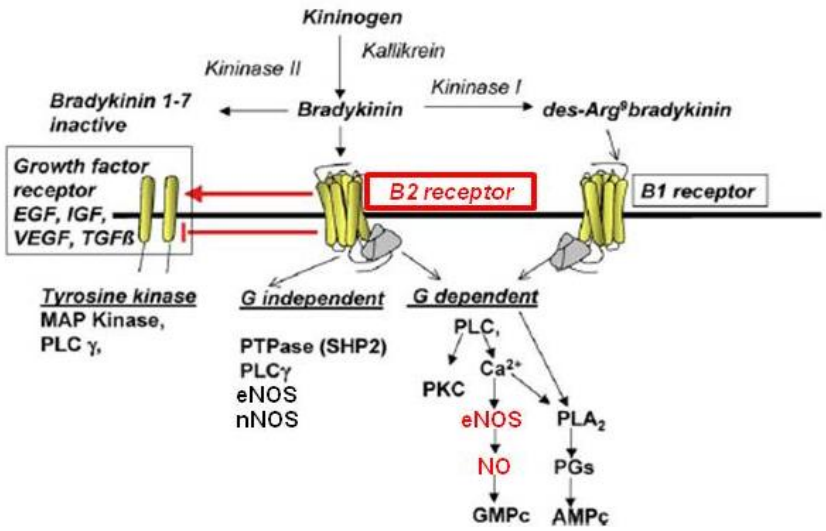

Figure 33. Overview of Kallikrein-Kinin system

Those particularly relevant to the author's research are highlighted in red.

See Couture and Girolami 2004 for abbreviations and further details.

1.2.5 ACE I/Dp and *Alu* sequences: A brief overview

The 287bp *Alu* sequence in the ACE gene giving rise to the ACE I/Dp (figure 27A) belong to the short interspersed elements (SINE) family. SINEs are composed of repetitive sequences ranging in length from 90 – 300 bps, which are a type of transposable element (TE) (Caporale, 2006). These account for the vast majority of genetic material in the human genome, making up approximately 45% (Caporale, 2006). *Alu* sequences are randomly spread throughout the human genome and constitute approximately 10% of the total genome (Hasler & Strub, 2006). Originally thought to be junk DNA, partly because they were situated in non-coding stretches of DNA, evidence is now suggesting they may "actually be major originators of genetic change and diversity, facilitating populations to adapt and evolve when changes occur in the environment" (Costa, 2008). It is also suggested that *Alu's* can be an alternative source for splicing when present in intronic (the case in the ACE I/Dp) regions of human genes (Hasler & Strub, 2006; Costa, 2008). Also they are an important point for regulation of gene expression by epigenetic mechanisms (Brohede & Rand, 2006; Costa, 2008). There is a very strong correlation between the ACE I/Dp and serum ACE activity levels (Rigat *et al.*, 1990; Butler *et al.*, 1999; Prasad *et al.*, 2000; Arcaro

et al., 2001). Danser et al., (1995) also demonstrated that the prediction on higher levels in ACE activity, in ACE genotypes lacking the 287bp *Alu*, held firm in cardiac tissue (Danser *et al.*, 1995).

However, as Ang2 is the product and effector molecule, of ACE, its activity or levels are of greater importance. It is generally reported that ACE activity is not correlated with Ang2 levels (Danser *et al.*, 1999; van Dijk *et al.*, 2000; Wang *et al.*, 2008). This statement may not hold true for a number of reasons: 1) As stated earlier (section 1.2.3) it cannot be stressed enough how difficult accurately quantifying Ang2 is, 2) The ACE has two catalytic domains, but the Cd is thought to be the only active Ang1-to-Ang2 converting domain and is the only domain active in the ACE DD genotype (van Esch *et al.*, 2008), 3) Even after accurate measurement of Ang2 this may not reflect the local concentrations within cells, especially as ATR1-bound Ang2 has a prolonged half-life of approximately 30 times that of circulating Ang2 (van Kats *et al.*, 1997), 4) It may not be the exact levels of ACE or Ang2 that are important but how efficiently Ang1 can be converted to Ang2, which has been demonstrated to be enhanced in the ACE DD genotype (Ueda *et al.*, 1995; Brown *et al.*, 1998b; Prasad *et al.*, 2000; San Jose *et al.*, 2009; Woods, 2009), 5) Increased shedding, which elevates ACE levels in circulation, has also been shown to be affected by the ACE I/Dp, with significantly higher circulating levels in those (rather strangely) individuals with the heterozygote ACE I/Dp (van Esch *et al.*, 2008), 6) Finally (as mentioned earlier), age, ethnicity, sex, and vascular health all effect (and confound) ACE expression and will thus circulating levels.

1.2.6 An overview of ACE I/Dp research and motivation

Hypertension was responsible for 13 million deaths in 2004 (WHO, 2009). 51% of the victims of a lethal stroke and 45% of ischaemic heart disease are directly attributable to hypertension. A healthy CVS is of paramount importance to reduce and prevent the chronic diseases plaguing modern societies.

Cardiovascular system and remodelling: Hypertension results from a detrimental remodelling of the CVS, brought on mainly by environmental factors; physical inactivity, smoking, excessive alcohol, excessive energy intake and poor dietary

choices of which the exact contributions are still debated. In short hypertension is *the major risk factor* for the development of CVD.

Maintenance of systolic blood pressure is essential to perfuse tissues and organs with adequate blood supply to deliver nutrients, oxygen and remove waste products while not damaging the blood vessels mechanically. Blood pressure is regulated by a complex interplay of many factors, including: hormones, central nervous system, heart rate/stroke volume and blood vessel structure (Schork, 1997). However, one of the most significant physiological changes that determine all vascular pathologies, especially hypertension, is vascular remodelling (Heeneman *et al.*, 2007). The vascular wall is exposed to a continuous flow of blood, which causes shear stress and luminal pressure (Heeneman *et al.*, 2007). Acute changes in shear stress and luminal pressure cause alterations in vessel diameter, which if caused by physical activity can be beneficial because the pressure impacts on dilated vessels. But chronically elevated shear stress and luminal pressure, together with inflammatory processes, result in structural changes, and ultimately in cardiovascular pathologies, chiefly hypertension and atherosclerosis (Heeneman *et al.*, 2007). Ang2 is one of the key molecules involved in the vasoconstrictory phenotype, which comprises vascular remodelling, including hypertrophy and inflammation (Jones & Woods, 2003). This anticipated relationship fostered a considerable interest in the Rigat's finding on the regulation of ACE activity by the ACE I/Dp. Was there really a genetic component that affected ACE activity? If there was then surely this would also result in an increased (D allele)/decreased (I allele) risk of developing cardiovascular diseases, especially those related to the development and progression of hypertension?

The cardiovascular link and ACE I/Dp: Individual studies have shown conflicting findings, but the majority of evidence points towards studies with positive findings showing an association of increased CVD risk with the D allele, see Sayed-Tabatabaei, (2006), and Agerholm-Larsen *et al.*, (2000) for a review and meta-analysis respectively (Agerholm-Larsen *et al.*, 2000; Sayed-Tabatabaei *et al.*, 2006). It is generally thought that any influence of the ACE I/Dp will be very small due to the extremely complex nature and development of CVD (Sayed-Tabatabaei *et al.*, 2006), which includes a combination of genetic and environmental factors (such as smoking, nutrition, stress, chemicals, and exercise).

Energy metabolism and substrate delivery: A person that has been regularly physically active over many years will certainly have a very different physiology including the vascular and muscle makeup than a person who has been sedentary all their life (see Figures 6, 10-12, 15, 16, 19, 21).

The vascular system and final delivery vessels are greatly influenced by the key [ACE] regulatory enzyme of a major vasoconstrictor, Ang2. These delivery vessels, in addition to transporting and removing oxygen and carbon dioxide respectively, provide important metabolic substrates that fuel muscle contraction. These substrates are critical determinants of sustained muscle work, which limits exercise performance (Holloszy *et al.*, 1998). With the onset of muscle contraction the resting vasoconstrictory normal state is overridden causing blood vessels to dilate and thus facilitate delivery of energy substrates to drive muscle work (Murrant & Sarelius, 2000). Evidence also suggests that the ACE I/Dp may have an important influence on substrate utilisation. Kinins (powerful vasodilatory molecules), which are degraded by ACE, increase Glut-4 translocation to sarcolemma and increase glucose transport (Dietze & Henriksen, 2008). The I-allele is associated with increased insulin sensitivity and increased uptake of glucose and glycogen stores (Vigano *et al.*, 2009). ACEI and AT1RB consistently relate to improvements in insulin sensitivity and progression to DM2 (Scheen, 2004; Muscogiuri *et al.*, 2008). BK increases tyrosine kinase activity, which increases IRS-1, which increases Glut-4 in SKM and glucose transport by increases in PI3 kinase, BK also stimulates phosphofructokinase (Muscogiuri *et al.*, 2008). ACEI (mirror the I-allele) and AT1RB consistently relate to better improvement in glucose disposal and insulin sensitivity (Muscogiuri *et al.*, 2008).

There is also some evidence that fatty acid delivery and metabolism may be importantly altered, due to the ACE I/Dp, although there are few studies investigating this. Total cholesterol and LDL levels have been shown to be higher in ACE DD genotypes, compared to ACE ID or ACE II genotypes (Kim, 2009). Although an earlier study found no difference in LDL between ACE I/Dp genotypes, but did find a trend for reduced levels of TAG (P=0.06) in ACE II genotypes compared to ACE ID or DD genotypes (Arcaro *et al.*, 2001).

The RAS system regulates vascular space, and any factor that contributes towards altered functioning (like possibly the ACE I/Dp) could have important consequences for both disease progression and human performance.

Influence of ethnicity: Ethnicity is an often over-looked confounding factor when studying disease, especially those that develop over decades, such as cardiovascular diseases. ACEI, which are used in first line treatment of hypertension, are less effective in reducing blood pressure in blacks than whites (VACSGoAA, 1982; Materson *et al.,* 1993) and that there may be a different mechanism in which ACEI lower blood pressure in blacks (Weir *et al.*, 1995). Studies have shown that there are differences in efficacy of ACEI between ACE IDp with 100 times more ACEI needed to block Ang1-Ang2 conversion in ACE II's vs. DD's (van Esch *et al.*, 2008).

South Asians have an increased risk of developing cardiovascular diseases, and DMII (Gupta *et al.*, 2006; Gholap *et al.*, 2011), respectively – and at a much younger age (Gupta *et al.*, 2006). A study on the ACE IDp with ethnicity suggested there was an interaction effect, which produced different vasodilatory responses in blacks versus whites (Gainer *et al.*, 2001). Also, the often quoted association between ACE activity levels and the ACE IDp (DD>ID>II) relates to Caucasian populations. For example, the ACE IDp is not associated with serum ACE activity in black South African populations (Payne *et al.*, 2007). It is often quoted in the literature that the allelic frequency of the ACE I/Dp is 1:2:1 (DD:ID:II). However, there is clear evidence that this is not the case across all populations (see Figure 31). There appears to be a distinctly increased frequency of the D allele in European populations of 0.55 (mean D allele frequency from 69 separate studies – Agerholm-Larsen *et al.,* 2000). There has also been a study which reported no significant correlation with ACE activity and the ACE I/Dp in African-American children, even though the ACE I/Dp frequencies were not significantly different from the white children in the study (Bloem *et al.,* 1996). Thus, studying mixed populations even if subjects appear to be from one ethnic population (without knowing family ethnic history) could potentially hide any ACE I/Dp association.

Human performance: Montgomery's original article (Montgomery *et al.*, 1998) commented on the significant increase in frequency of the I allele in mountain climbers and (in a second cohort) an increase in duration of repetitive elbow flexion (following a physical training programme) – also in I allele carriers. They concluded

there may be an association with the I allele and improved endurance performance. Subsequent studies have agreed (Gayagay *et al.*, 1998; Myerson *et al.*, 1999; Alvarez *et al.*, 2000; Scanavini *et al.*, 2002; Collins *et al.*, 2004; Hruskovicova *et al.*, 2006; Thompson *et al.*, 2006). A number also shown no association (Taylor *et al.*, 1999; Rankinen *et al.*, 2000b; Frederiksen *et al.*, 2003; Scott *et al.*, 2005; Day *et al.*, 2007; McCauley *et al.*, 2009; Papadimitriou *et al.*, 2009) or the opposite, i.e. the association of the D allele with endurance performance (Zhao *et al.*, 2003; Lucia *et al.*, 2005; Amir *et al.*, 2007; Muniesa *et al.*, 2010). See a review article by Puthucheary, et al. (2011) for a further insight into the types of studies conducted on the ACE gene and human performance, since the link was first proposed (Puthucheary *et al.*, 2011).

Rather than looking for associations (which many of the studies in the current literature did) with the ACE I/Dp and human performance (endurance or strength) or trainability it may be its influence on the ACE product, Ang2, which is of most interest. Evidence suggests that ACE I/Dp-dependent modulation of exercise performance is mediated by the effects of the ACE product, Ang2, on capillary and fibre growth in muscle (Greene, 1998; Schaufelberger *et al.*, 1998; Booth *et al.*, 2002; Zhang *et al.*, 2005; de Resende & Greene, 2008).

1.2.7 Aims and Objectives of the research project

The aim of this research project was to investigate the effects of endurance exercise on substrate metabolism and to examine whether the ACE I/Dp genotype (via its link to angiotensin 2 levels) was associated with metabolic responses to exercise. There were four main objectives:

1. To compare physiological phenotype of endurance trained versus sedentary young men (chapter 3).
2. To examine whether endurance trained and sedentary young men show different metabolic responses to a single bout of exercise (chapter 3).
3. To examine whether the ACE I/Dp genotype is associated with energy metabolism in a British population (chapter 4).
4. To examine whether the association between ACE I/Dp genotype and energy metabolism that was observed in the British population was also evident in a cohort from Switzerland (chapter 5).

Chapter 2

Methods

2. Methods

2.1. Experimental Methods

Ethical approval (see appendix 1): The studies conformed to the latest revision of the Declaration of Helsinki and were approved by the ethics committee of the Faculty of Science and Engineering at Manchester Metropolitan University. All participants provided informed, written consent prior to participation in the study and were free to withdraw from the study at any time if they so wished.

ACE I/Dp Genotyping

DNA was extracted from mucosal swabs using a custom designed protocol as follows: Mucosal swabs were collected with ear buds, extracted with 800 µl of methanol and vortexed, soaked then briefly centrifuged, and then left to evapourate. When all the methanol has evapourated the pellet is frozen at -80^{o}C overnight (this introduces breaks into the DNA, which enhances the accessibility of the primers to the single complementary sequence in the large chromosome). The next day the pellet was resuspended in 100 µl of sterile water, soaked, vortexed, heated for 15 minutes then centrifuged at 10, 000 rpm for 10 minutes. Supernatant (containing DNA) was transferred into aliquots and stored at -20^{o}C until needed. ACE I/Dp genotyping was performed by polymerase chain reaction (PCR) as described by Evans et al. 1994 (Evans *et al.*, 1994). The primers corresponded to those established previously for the identification of the ACE I/Dp genotype (for details see Genbank number X62855). Detection of the 66 bp amplicon, specific for the I-allele in intron 16 of the ACE gene was achieved by a combination of ACE1 (5'-catcctttctcccatttctc-3'), and ACE3 (5'-atttcagagctggaataaaatt-3') primers. ACE2 (5'-tgggattacaggcgtgatacag-3') and ACE3 (5'-atttcagagctggaataaaatt-3') primers were applied to detect the 83 bp amplicon specific to the absence of the insertion sequence (i.e. the D-allele).

PCR reactions were run with a mix of the three primers using SybrGreen Master Mix (Applied Biosystems) on a Biorad DNA machine controlled by the MJ Opticon Monitor software (Biorad). The protocol involved 45 standard cycles of denaturing at 95^{o}C for 15 seconds followed by annealing and extension at 55^{o}C for

one minute. Amplicon identification followed using a melting curve analysis between a temperature range of 65^{o}C to 85^{o}C. The amplified sequence identity for the ACE I-allele and ACE D-allele was validated by sequencing of the PCR products with the specific primers (Microsynth, Balgach, Switzerland). The presence of the short amplicon for the I-allele was identified by a lower melting temperature (73.07^{o}C ±0.15) compared to the amplicon for the longer D-allele (74.91 ^{o}C ±0.17). The difference in melting temperatures between the two alleles was highly significant ($p<2.53^{-38}$, T-Test, n=28). Samples with poor signal to noise ratio were re-run in separate reactions with the specific primer pair for amplification of either the I-allele or D-allele.

One-legged cycling peak power/oxygen uptake test (VO_2peak test)

Participants will complete two incremental exercise tests (peak power/VO_2peak tests) to the limit of tolerance for one-legged cycling exercise (Davies & Sargeant, 1975) on the electrically braked cycle ergometer (Jaeger ergo-line 2000, Germany). These two tests will be separated by at least 72 hours. The non-exercised leg will rest on a fixed chair and that corresponding ergometer pedal removed. Saddle and handlebar positions will be adjusted for each participant and the positions recorded to allow accurate replication of the test conditions in the acute endurance test (see below). The exercising foot was then taped securely in place, with strong electrician's tape. Each participant was instructed to concentrate on driving down with the leg and not pulling up, plus they were also instructed to focus on generating the power from their exercising leg and not moving their body – especially near the end of the test.

Following a four minute warm-up period at of increasing intensity (per minute) from 10 to 40W, cycling with one leg, the external work rate was increased by a further 5W every 10 seconds. A pedal frequency of 80 rev.min^{-1} was maintained throughout the exercise period. The test was terminated when the pedal rate fell consistently below 60 rev.min^{-1}, despite strong verbal encouragement. Pulmonary gas exchange was measured breath-by-breath (Cosmed K4b2, Italy) and VO_2peak and peak power (W) was determined using standard techniques. Peak power was taken as the highest value recorded during either one-legged test. One-legged VO_2peak was recorded as the highest 30 second average oxygen uptake during either of the two

tests. Heart rate was monitored throughout with a Polar heart rate monitor (Polar Electro, Kempele, Finland).

Immediately following termination of the test, the participants were encouraged to perform an active recovery by cycling at a very low external work load using two legs. This should have reduced sensations of local muscle fatigue and light-headedness.

Two-legged cycling oxygen uptake VO_2max

Each participant completed (at least) two incremental exercise tests to the limit of tolerance for two-legged exercise on an electrically braked cycle ergometer (Jaeger ergo-line 2000, Germany). Each test was separated by at least 24 hours. Following a four-minute warm-up period at 40W, the external work rate was increased by a further 5W every 10 seconds until volitional exhaustion. A pedal frequency of 80 $rev.min^{-1}$ was maintained throughout the exercise period. The test was terminated when the pedal rate fell consistently below70 $rev.min^{-1}$. Pulmonary gas exchange was measured breath-by-breath (Cosmed K4b2, Italy) and the gas exchange threshold and VO_2max was determined using standard techniques. VO_2max was recorded from either of the two tests with the highest 30 second average oxygen uptake. Heart rate was monitored throughout with a suitable device (Polar Electro, Kempele, Finland). Saddle and handlebar positions were adjusted for each participant and the positions recorded to allow accurate replication in each test. Immediately following termination of the test, the participants were encouraged to perform an active recovery by cycling at a very low external workload. This should have reduced sensations of local muscle fatigue and light-headedness.

Respiratory Expired Ratio

Pulmonary gas exchange was measured breath-by-breath (Cosmed K4b2, Italy). To calculate the RER at the beginning of the test a 15 second time period was averaged at approximately 2 minutes into the exercise bout (warm-up period at very low resistance) for both one- and two-legged tests. To calculate the RER at maximum exertion an average of 15 seconds was taken approximately 15 seconds from the end

of the test. Heart rate was monitored throughout with a suitable device (Polar Electro, Kempele, Finland).

Quadriceps muscle volume

Magnetic resonance imaging (MRI) was used to measure muscle volume of the quadriceps femoris muscle group of the exercising leg (one-legged VO_2peak test) using a 0.2-T G-scan MRI scanner (Esaote, Genova, Italy). Participants were scanned in the supine position with the leg fully extended and relaxed, and held firmly (but comfortably) in place.

Serial cross-sections (each measuring 3.1 mm in thickness, with a 18.6 mm inter-slice gap) were acquired from the lateral femoral condyle to the greater trochanter using a turbo 3-D T1 protocol. Total volume (cross sectional area x thickness) of the quadriceps muscles was analysed in each slice using image processing software (OsiriX version 3.3.2, 32-bit). Total quadriceps muscle volume was estimated based on the integration of volume from each slice and the inter-slice gap using a modified method described by Seynnes et al. 2008 (Seynnes *et al.*, 2008).

Body fat percentage

We measured substrates involved in exercise metabolism, and fat plays an important role in this, hence we wanted to accurately measure body fat percentage. Dual Energy X-ray Absorptiometry (DEXA) – Lunar Prodigy Advance (GE Healthcare, Waukesha, Wisconsin, USA) – was used to assess each participant's whole body fat percentage. There is a theoretical risk involved by the radiation imposed. In reality, that risk is rather low, as total body equivalent dosage is expected to be 0.4 microSievert. This could be compared to the background radiation of more than 2000 microSievert per year, or to the additional radiation of 10 microSievert per hour of air flight.

Each participant removed all their clothing apart from their underwear (shorts). They were then positioned, per whole-body composition analysis protocol, appropriately on the DEXA scanner and ankles and knees secured in place to ensure actual whole-body analysis. A QA check was performed before each scan – only upon passing this check did the scanning proceed.

Acute cycling endurance test

The cycle ergometer (Jaeger ergo-line 2000, Germany) was set-up according to measurements recorded during the one-legged VO_2peak test, including the important securing of the exercising leg/foot to the cycle pedal. There was a warm-up period of 8 minutes, which allowed for a progressive increment in Watts – the actual increment per minute varied from 10 – 30 W depending on the steady-state power output calculated. After 20 minutes cycling one-legged at 60% peak power the external work load (W) was increased by 5 W every 10 seconds. Pulmonary gas exchange was measured breath-by-breath (Cosmed K4b2, Italy) and VO_2peak and peak power (W) were determined using standard techniques. Heart rate was monitored throughout with a Polar heart rate monitor (Polar Electro, Kempele, Finland).

Immediately following termination of the test, the participants were encouraged to perform an active recovery by cycling at a very low external work load using two legs. This should have reduced sensations of local muscle fatigue and light-headedness.

Serum metabolite measures

Total cholesterol, HDL, triglycerides, glucose and ketones were measured at rest (fasted) and immediately following exercise with a CardioChek ST Analyzer (Test Medical Systems @ home). LDL was also calculated by using the Friedewald formula: LDL = total cholesterol – HDL – triglycerides/5 (Friedewald *et al.*, 1972). Blood was sampled via finger-pricks or drawn from the upper arm via the median cubital arm (for five participants only).

Participants were seated before blood sampling begun. If blood was drawn via finger-pricks then the finger would be cleaned with an alcohol swab and left to air dry. A self-projecting/retracting lancet would then pierce the skin. Blood would gently be massaged (if needed) and the first drops wiped away. Gentle massaging of the finger would continue (if needed) and the blood would be drawn up into to heparinised capillary tubes and fixed in place (using blu-tak) on a rocking plate (to further ensure the sample did not coagulate). Another two capillary tubes collected blood (30ul x 3 tubes) and were placed onto the rocking plate. Sometimes a fresh finger-prick was

required to obtain sufficient blood. Upon collecting enough blood the appropriate metabolite analysis strip would be placed into the CardioChek ST Analyzer and 15ul of blood dispensed (with a special capillary plunger) onto the analyzer window.

Muscle biopsies:

Technique One (Chonchotome)

A small (~ 50-100 mg) biopsy was taken from the *vastus lateralis* muscle of the non-exercising leg, of the quadriceps, at 50% of femur length as assessed by prior ultrasound analysis (an acetate was used to draw around the knee cap and identifying marks – moles/scars – also drawn on to ensure accurate placement for taking biopsy). Participants were asked if they have ever had an adverse reaction to a local anaesthetic; if not local anaesthetic (2% Lidocaine) was infiltrated under the skin. After the skin had been shaved and sterilized an incision in the skin was made to the chonchotome to sample a piece of the *vastus lateralis* muscle. Once the sample was taken it was rapidly wiped on a sterile surface to remove excess blood and cut in two pieces. The first piece (for molecular analysis) was rapidly placed in iso-pentane (which itself was submersed in liquid nitrogen) to help reduce the chances of damage to the muscle ultrastructure (which can happen if rapidly cooled/placed straight into liquid nitrogen) and then liquid nitrogen. The second sample was immediately mounted on cork (orientating the fibres longitudinally) using Tissue-Tek, and then the above protocol followed. All samples were then stored at -80^{o}C until later analysis.

Technique Two (Fine-needle)

Biopsies two and three (30 minutes and 8 hours post exercise) were performed with a spring-loaded and reusable instrument; the ACE-Onecut Disposable Biopsy (14 Gauge x 150 mm, product code ONE-141502, UK BIOPSY Ltd, Great Britain). Both these samples were for molecular analysis and the same protocol was followed as in technique one.

Capillary Density Determination

Sections were stained with lectin to identify capillaries (Ahmed *et al.*, 1997). Sections were left to dry (once removed storage at -80^{o}C), fixed in acetone (4^{o}C) for 15 minutes, incubated in HEPES-buffer for 10 minutes, incubated in peroxide for 30 minutes (to reduce background staining), incubated in HEPES-buffer for 5 minutes, incubated in lectin (Ulex Europeus) in HEPES 1% BSA (2000ug/ml). The capillaries were visualised using a Vectastain ABC and DAB substrate kit (Vector Laboratories, Peterborough, UK) before mounting in gelatine-glycerol.

The capillaries were analysed using the method of capillary domains (Degens *et al.*, 1992). This method not only takes into account the overall indices of capillary to fibre ratio (C/F) and capillary density (CD), but also takes into account the heterogeneity in capillary spacing. Photomicrographs were taken of cross-sections of the *vastus lateralis* muscle (scale?) and the outlines of the muscle fibres together with the location of each capillary were digitised on a calibrated digitising tablet (Model MMII 1201, Summagrahics Digitisers, Austin, Tex, USA. The overall capillary density (CD) was defined as the number of capillaries mm^{-2} of tissue. Capillary domains, defined as the area surrounding a capillary delineated by equidistant boundaries from adjacent ones, were constructed and their surface area calculated. From the overlap of the domains with muscle fibres, the local capillary to fibre ratio (LCFR) and capillary fibre dendity (CFD) were obtained. The LCFR for a fibre was defined as the sum of the fractions of each domain area overlapping the fibre. This variable has the continuous distribution and is more sensitive than, for example, capillaries around a fibre. It importantly also allows determination of the capillary supply to a fibre even when it lacks direct capillary contacts. CFD, which is LCFE divided by the FCSA, provides the capillary density of that fibre and is expressed as the number of capillaries mm^{-2}.

Fibre type percentage determination

Immunohistochemical determination of fast, slow, and hybrid fibre types was carried out on 10 μm cryosections using monoclonal antiobodies specific for fast myosin (My-32, Sigma) or slow myosin (MAB1628, Chemikon).

Slides with 10 μm cryosections (from *vastus lateralis* muscle) were removed from -80^{o}C freezer and left to thaw. Sections were then fixed in acetone, quenched with peroxidise (3% H_2O_2 in Methanol), washed with PBS, blocked with 3% BSA/PBS, incubated with primary monoclonal mouse antibody (specific for fast/slow fibre), washed in PBS, reacted with peroxidise-conjugated anti-mouse IgG (A-2304 Sigma, 1:2000 in 0.3% BSA/PBS), washed in PBS, detected immunoreactivity with substrate AEC (Sigma Chemicals, Buchs, Switzerland), incubated in sterile water to stop, counterstained with hematoxylin, placed in tap water to develop, washed in PBS, and before allowing sections to dry Aquatex was added and coverslide placed over sections.

Gene expression analysis

RNA isolation and real-time PCR: Cryosections from VL muscle were sampled in frozen tubes, and RNA was extracted using a modified Qiagen protocol (purification of total RNA from animal tissues) as follows:
25 μm cryosections were cut (10mm^3 per sample) on a cryostat, and sections transferred to 2.0 ml cryo-vial tubes and stored at -80^{o}C. After adjusting to -20^{o}C , 333 μl of lysis buffer (RLT-buffer, Qiagen AG, Basel, Switzerland; and β-mercaptoethanol) pre-heated to 45^{o}C was added and the muscle sections immediately homogenized (on ice) with a rotor-stator homogenizer (Polytron PT1200, KINEMATICA AG, Lucerne, Switzerland) for 10 seconds, 567 μl of DEPC water was added (washing around homogenizer tip to collect all sample) and immediately homogenised, finally 100 μl of proteinase K was added to the sample. The sample was then left at 45^{o}C for 90 minutes for proteins to digest. The sample was then centrifuged for 10 minutes at 7500 rpm and supernatant transferred into sterile 15 ml tubes, 1 ml of RLT buffer + β-mercaptoethanol (vortex) then 1 ml of EtOH – 4^{o}C (vortex) was added, this solution was then added (in 700 μl volumes) to RNeasy columns and centrifuged at 13, 000 rpm for 15 seconds, the column had a final wash and spin (at 13, 000 rpm) with RW1 buffer after which 10 μl of DNase + 70 μl RDD buffer was added to the column and left to digest for 30 minutes. Another 350 μl of RW1 buffer was applied to the column and centrifuged at 13, 000 rpm for 15 seconds, two success washes of the column with 500 μl of RPE buffer (and centrifuge at 13,

000 rpm for 15 then 120 seconds) were applied. RNA from the column was then eluted into RNase free tube with two success applications of 50 µl of DEPC water – applied to the middle of the column filter – incubated for 60 seconds and centrifuged for another 60 seconds at 13, 000 rpm. 10 µl of sodium Acetate (pH 5.2) then 250 µl EtOH (vortex and spin at 13, 000 rpm for 120 seconds) was added to the sample and left overnight at -20^{o}C. Sample was centrifuged at 10, 000 rpm for 30 minutes (at 4^{o}C) then supernatant removed. The remaining pellet was then very carefully washed with 70% EtOH (previously stored at -20^{o}C) centrifuged at 10, 000 rpm for 5 minutes (at 4^{o}C), the supernatant removed, RNA pellet was then left to air dry for approximately 60 minutes. 3 x 2 µl of DEPC water were added next to the pellet and gently tapped into the pellet, 1 µl was then removed and diluted (1:9, for RNA quantification using a spectrophotometer, absorption at A_{260}). 5 µl of dissolved RNA was left in the sample. 100 ng RNA was reverse transcribed and subjected to real-time polymerase chain reaction (RT-PCR) for selected transcripts – see table 18 chapter 3. Reactions were run with Sybr Green master mix (Applied Biosystems) on a Biorad DNA machine controlled by the MJ Opticon Monitor software (Biorad). Due to a low quantity of RNA, used per reaction (3 ng), we altered the standard protocol (50 standard cycles 15 sec 95ºC, 1 min 55ºC) to include an extra step, after annealing (1 min 55ºC), of 15 sec 75ºC to reduce non-specific binding – this was performed after a hot start on same amounts of cDNA. Primers were used as established (Zoll *et al*., 2006) or designed with the online NCBI Primer-Blast primer design tool (Rozen & Skaletsky, 2000) and synthesized at Sigma-Genosys. Signals were expressed per input amount of cDNA using the delta cycle threshold (Ct) method and transcript signals were standardized to 28S rRNA. Amplification was assessed by a combination of analysis of melting curve and Ct. Briefly, for amplification to be deemed successful there must have been a single clearly identifiable melting peak together with a clear exponential increase (sigmoid curve) and plateau (although a plateau may not be seen for very low abundant cDNAs that show up/amplify late). All primers passed both these conditions apart from AT2R, BK2R and HMWK.

Muscle Metabolite Analysis – Mass Spectrometry

This analysis was carried out in collaboration with Prof. Royston Goodacre from the University of Manchester. The author's part in this specific part of the study was limited to the initial muscle biopsy preparation before passing over to University of Manchester who did the actual metabolite analysis (as described below). The protocol for extraction of muscle metabolites was established in preliminary experiments. Based on these results the following protocol was adopted.

Sample preparation:

Extraction SOP (Yu Lin, 2007):

Washed samples 2-3 x in 0.85% NaCl to remove residual blood, keeping sample (30 or 40 mg FW fine ground in ball mill) on ice, add 690 µl of CHCl3 : MeOH (1 : 1) (All solvents of HPLC grade or better), vortexed for 10 sec and shake at 4^{o}C (cold room) for 15 min, centrifuged at max speed for 5 min (cryofuge at 3^{o}C) and remove supernatant to a clean 2 ml tube on ice, 310 µl of H_2O added, vortexed and then centrifuged at max speed and 3^{o}C for 5 mins to aid phase separation, polar extracts were normalised for sample weight differences by amending the volume of extract dried down (please see Table metabolomics below), Aliquots of the polar phase to a clean labelled 2 ml tubes (take care not to disturb interphase), the remaining polar phases were pooled to produce a QC sample, to the polar extract 100 ul IS2 (to correct for analytical error) was added, polar samples were dried (fully) in a speed vacuum concentrator for ~ 4-6h, and stored at -80 ^{o}C, non-polar samples are directly flash frozen in liquid nitrogen and stored at -80 ^{o}C in CHCl3.

Table metabolomics

Pre-Exercise samples					Post-Exercise samples				
Randomised Extraction Order	Sample / test subject number	UMAN Unique sample number	Sample weight (mg)	Volume of 1ml extract to lyophilise (uL)	Randomised Extraction Order	Sample / Test subject number	UMAN Unique sample number	Sample weight (mg)	Volume of 1ml extract to lyophilise (uL)
1	25	1	24	375.0	1	25	18	25	360.0
3	27	2	32	281.3	3	27	19	31	290.3
4	62	3	27	333.3	4	62	20	26	346.2
6	24	4	18	500.0	6	24	21	27	333.3
7	26	5	45	200.0	7	26	22	22	409.1
8	40	6	38	236.8	8	40	23	26	346.2
9	37	7	39	230.8	9	37	24	26	346.2
10	3	8	20	450.0	10	3	25	29	310.3
11	4	9	21	428.6	11	4	26	20	450.0
12	42	10	18	500.0	12	42	27	20	450.0
13	53	11	33	272.7	13	53	28	21	428.6
16	51	12	22	409.1	16	51	29	26	346.2
17	57	13	18	500.0	17	57	30	21	428.6
18	11	14	18	500.0	18	11	31	39	230.8
19	45	15	19	473.7	19	45	32	26	346.2
20	49	16	34	264.7	20	49	33	25	360.0
21	56	17	15	600.0	21	56	34	27	333.3

Sample analysis:

Focused upon LC-MS based techniques, as this has the potential to reveal a greater depth of information than Gas Chromatography-MS (GC-MS).

Samples were analysed using the Thermo LTQ Orbitrap MS system since it offers the greatest sensitivity. Metabolites were recovered in the non-polar and polar phase. After derivatization the non-polar phase (chloroform) was subjected to direct infusion mass-spectrometry (DIMS). The polar phase was dried down, resuspended in equal proportion of solvent (per mg of tissue) and subjected to separation by liquid chromatography-mass-spectrometry in positive (LCMS_ESI+) and negative (LCMS_ESI) electrospray ionisation mode.

Polar samples

Polar samples (methanol and water phase of the extract) were reconstituted in a small volume of water (50ul) to maximise sample concentration and run on a Waters UPLC in a gradient from high aqueous to high organic (A: Water acidified with formic acid and B: Acetonitrile acidified with formic acid). Data were collected on the Thermo

LTQ-Orbitrap in both ESI+ve and ESI-ve mode (follwoing HUSER-MET serum protocol), the Orbitrap was operated to minimise sample fragmentation so that we hope that the masses we detect originate from whole intact metabolites.

Non-polar samples

Non-polar samples (chloroform phase of the extract) were diluted to an appropriate level and were analysed directly. The samples rather than being subjected to UPLC, were directly infused into the Thermo LTQ Orbitrap operated in ESI-ve mode , since the samples are not diluted by LC eluents, this gives nice rich profiles of triglycerides, phospholipids and fatty acids.

Paired samples (i.e. same participant pre and post exercise) were separated in consecutive runs of the calibrated equipment. Control experiments demonstrated a correlation of z of such paired experiments.

Data processing: Because no internal standard was used data normalisation was indicated to allow the comparison of relative signals resulting from the count of peak signals in mass spectrometry. Peaks with signals below 12.5 x the standard deviations of blank samples were set to zero. Data were normalized to the median peak ratio as described (Wang *et al.*, 2003). In brief, a reference profile was constructed for pre- and post-exercise samples from the median of values for each peak. Subsequently, for each sample profile the fold difference of each peak versus the respective peak of the reference profile was estimated, values with a denominator of "0" were removed and the mean fold difference of all peaks was determined. This value was used as normalization factor to adjust the signals of each experimental sample. Then the revealed normalized values were related to the mean of pre-exercise signals from the respective training cohort.

Metabolites for non-polar mass spectral features giving rise to the peaks of interest (Tables 15-17) were putatively identified by submitting the accurate mass information to the LipidMaps database (http://www.lipidmaps.org/).

Chapter 3

Exercise Alters the Human Muscle Metabolome

3.1. Introduction

High intensity exercise greatly increases energy expenditure and metabolic rate must increase to provide the ATP necessary for continuation of muscle contraction. Oxygen consumption can increase by more than 10-fold, from resting values of around 0.3-0.5 L/min to values in excess of 3-5 L/min at maximal oxygen uptake. During prolonged low-to-moderate intensity exercise, it is lipid oxidation that provides the majority of the ATP, particularly in endurance trained people, but as exercise intensity increases to levels beyond 60% VO_2max, lipid oxidation decreases and the reliance on glucose as substrate increases (Venables *et al.*, 2005). These substrates can be sourced locally from muscle TAG and glycogen stores for lipid and glucose, respectively. They can also be supplied to the contracting skeletal muscles from adipose tissue and liver glycogen reserves from the circulatory system (Frayn, 2003).

With recent advances in technology and metabolomics it has become evident that numerous complex signalling and molecular events occur in response to exercise and many of these can be observed in blood serum (Yan *et al.*, 2009; Lehmann *et al.*, 2010; Lewis *et al.*, 2010). These metabolic changes are undoubtedly necessary to effectively meet the challenge associated with the metabolic disturbance of intense, prolonged physical activity. As regular endurance exercise can lead to extensive phenotypic changes to skeletal muscle and other body systems, the precise response, is likely to differ between untrained and endurance trained individuals. The general endurance-training adaptations that occur within skeletal muscle have been well characterised, and include increased lipid oxidation; volume density of intramyocellular lipids and mitochondrial enzymes. There are also adaptations that occur within the cardiovascular system, such as increased capillarisation; blood volume and stroke volume (Rosler *et al.*, 1986; Braun, 1991; Convertino, 1991), and hypertrophy of the cardiac muscle itself. These adaptations result in altered metabolic profile during exercise (i.e. higher lipid oxidation) and enable physical activity to be sustained at higher power output for longer durations. With extensive aerobic conditioning (training) skeletal muscles can improve their capacity for fat oxidation {Ramos-Jimenez, 2008}. Increases in volume density of intramyocellular lipids, and components of the respiratory pathway [extending from muscle organelles (i.e. mitochondrial volume density) to cardiovascular parameters (i.e. capillary density,

stroke volume)] all contribute to these ameliorations in endurance trained participants (reviewed in Keins, 2006). At the whole-body level this manifests in increased maximal oxygen uptake with a concomitantly reduced respired expiratory ratio (RER) at a given exercise intensity (Ramos-Jimenez, 2008). RER is an indirect method (gas analysis at the mouth) commonly used to estimate the respiratory quotient (RQ), which is the ratio of oxygen consumed to carbon dioxide expired. In steady state RER=RQ. However, as RQ reflects the usage/removal of oxygen and carbon dioxide respectively at the cellular level, during maximal exertion external respiration (measured during RER) will not match cellular respiration. Put another way, as RQ is measured at the local level, changes in buffer systems of the upstream elements of CO_2 and O_2 transport in the vascular distort the equilibrium.

Metabolomic studies have identified significant differences between trained and untrained individuals in the changes that occur in response to an acute exercise bout. For example, it is possible to differentiate key intermediates of glucose, lipid and amino acid metabolism following a bout of exercise and identify differences in these responses between trained and untrained individuals (Yan *et al.*, 2009; Lewis *et al.*, 2010). The metabolomic approach further identified differences between people of different skill levels and training status of the athletes, indicating that the subtle differences between trained and highly trained people can be detected. These metabolic differences were evident despite there being no notable differences between groups in the traditional biochemical markers of exercise, such as haemoglobin, testosterone, and creatine kinase (Yan *et al.*, 2009; Lewis *et al.*, 2010).

In the present study, it was hypothesised that a metabolomic approach would reveal changes in novel lipid species after an exhaustive bout of endurance exercise in SKM. It was further hypothesised that such changes would differ between untrained and endurance-trained individuals.

3.2. Methods

3.2.1 Experimental Design

The study design was intended to identify novel physiological differences within the muscle and cardiovascular systems between endurance trained and untrained people.

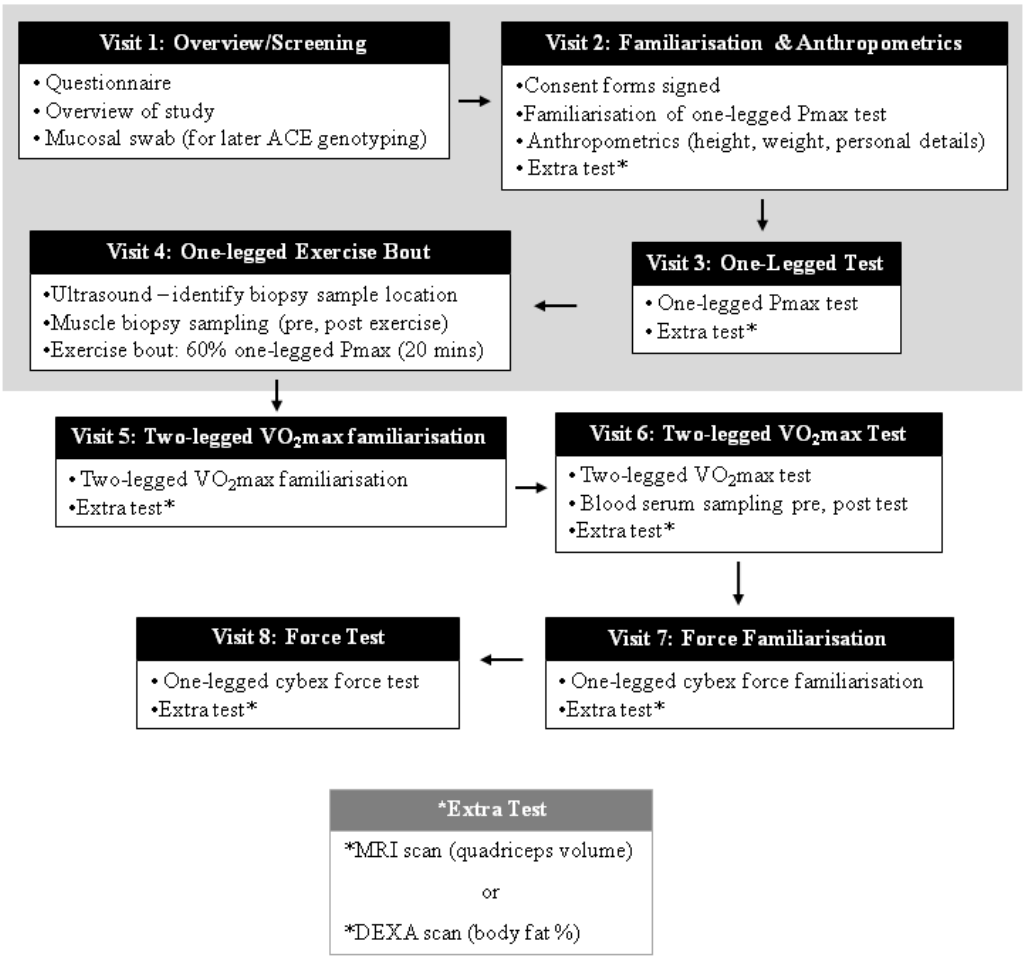

Figure 34. Outline and flow of tests used in study

Extra tests required no effort on participants part and could be conducted at any visit where indicated. The visits in the grey shaded area (visits 1 to 4) must be done in that order, visits 7 and 8 could come before 5 and 6.

There were many tests involved, requiring an absolute minimum of eight visits. Please refer to Figure 34 for an overview of the tests and order they were conducted per participant. Due to the complex nature of the study, which is studying the potential

interaction of many systems levels within a whole-organism (human) it was necessary to collect data from these different systems. Below is a description of why each test was chosen, problems encounted and development of tests:

Rationale for tests

Single-legged exercise was used to 1) maximally tax aerobic energy metabolism in contracting muscle without the central limiting factors imposed by the cardiovascular system that affects exercise of multiple body segments, 2) to allow the collection of a muscle biopsy from the non-exercising leg thus reducing the acute interference of this intervention on exercise performance and muscle metabolites. There were two different one-legged endurance tests: the main one being the acute one-legged exercise test where muscle biopsies are taken (visit 4 – the key visit). In order to ensure each participant exercised at the same relative intensity their one-legged Pmax would need to be calculated (visits 2 and 3). The steady state peak power output of 60% was chosen based (one-legged exercise bout – visit 4) research produced by Jamie McPhee and conversations with him – as this was a very novel area (McPhee *et al.*, 2010). The 60% intensity has also been piloted on volunteers (not any of the participants) and was deemed to be the maximum power output that could be reasonably maintained by one-leg but would also be great enough to challenge the working muscle. To ensure the local muscle would be maximally stressed a ramp was introduced at the end of the 20 minutes of steady state one-leg exertion. Previous studies have shown that during two-legged exercise the training intensity experienced by the local musculature differs from person to person (McPhee *et al.*, 2009; McPhee *et al.*, 2010). The single-legged acute exercise test (visit four) was performed early in the morning after participants had fasted over-night, to control for any dietary effects and to impose an additional challenge to metabolic control, as metabolism during moderate exercise is mainly driven by lipid oxidation in the fasted state (De Bock *et al.*, 2005).

A single-legged force test was also performed as were were interested to see is there were force differences between the participants who were trained and untrained.

A whole body DEXA scan was performed to assess body composition (mainly body fat percentage) and magnetic resonance imaging (MRI) was used to estimate quadriceps mucle volume. Evidence suggests that those people who regularly perform

physical activity (especially of the endurance type) will have reduced body fat and increased muscle mass – especially in the quadriceps – in essence these tests should confirm our conclusions about the training status of the participants. The order of the first four visits is vitally important – as we want each participant to do as little additional exercise as possible. Although very small the two extra two-legged VO_2max tests may start having an exercise effect on the untrained participants. Also, it's notoriously difficult to get participants to give muscle biopsies due to the invasive nature. There were a number of tests performed that were of interest to the participants, namely: VO2max, DEXA (body fat %) and serum blood measures. The most important visit for the study was the mucle biopsies – to keep participants motivation up we ensured those tests they were interested in came after the one that was key to our research. Also, participants were not told of there results from each test until all tests were completed, again to ensure they completed all tests.

The two-legged maximal oxygen uptake (VO_2max) cycling test was used to assess general whole-body aerobic fitness and is a well established measure – plus is was used to corroborate whether a participant was deem endurance trained or untrained. Blood samples were obtained before and after the two-legged cycling test to assess the effect of exercise on serum metabolites. Muscle biopsies were taken before and on completion of the single-legged exercise test (visit four), at 30 minutes and eight hours. We chose these two times because 30 minutes was the quickest time that a muscle biopsy could be taken following cessation of the exercise bout, .e.g. it takes about 10 minutes for the participant to get off the bike, compose themself and then lie down on the physio plinth, then it takes about 10-15 mintues for the doctor to prepare the area on the leg for the muscle biopsy to be taken (area to be cleaned, marked, and aneathestic to work). Eight hours was the time chosen for the last muscle biopsy as this is the time-point where important gene transcripts, relating to energy metabolism, have previously been identified as being up-/down-regulated (Flueck & Hoppeler 2003; Booth & Neufer 2005) – this muscle biopsy was not being used for metabolomic analysis. By utilising a one-legged exercise paradigm we could be more confident that all participants in these studies were performing work at similar relative intensities of their localised peak aerobic capacity (McPhee *et al.*, 2009).

Main serum metabolites (glucose, triglyceride, high- and low-density lipoproteins, total cholesterol and ketones), and the entire collection of non-polar metabolites, were

assessed in *m. vastas lateralis*. Capillary density and fibre types were also analysed to ascertain if these important local factors were significantly altered between the two cohorts.

3.2.2 Participants

Twenty healthy males (with no known medical contraindications) aged 19 – 38 participated in this study. Females were excluded due to potential differences in energy metabolism (e.g. greater usage of lipids during endurance exercise), plus we would have needed to controlled for each females menstral cycle, which was deemd to logistically difficult. Exclusion criteria included smoking, long-term ill health, female, and not within the study age range (18 – 39). Participants were deemed to be trained if they had a VO_2max >50ml/min/kg unless they deemed themselves unfit and sedentary or had indicated, by questionnaire and orally, the only activity they did was walking. This was the case of two of the untrained participants who had a VO_2max > 50ml/min/kg. The number of participants is on the lower side, when studying a gene variant. However, these numbers have been deemed acceptable for studies of a similar invasive nature (e.g. muscle biopsies, blood, maximal tests) in many other studies. Plus, the detailed analysis of many linked system levels allows for a finer resolution.

The methodology is listed below. A full description of study methodology can be found in the methods chapter (2).

3.2.3 List of all Tests/Measures (see methods chapter for description)

One-legged cycling peak power/oxygen uptake test (VO_2peak/Pmax test)
Two-legged cycling oxygen uptake VO_2max
Quadriceps muscle volume (MRI scan)
Body fat percentage (DEXA scan)
Acute cycling endurance test
Serum metabolite measures
Muscle biopsies
Capillary density determination

Fibre type percentage determination
Metabolome Profiling
Respiratory exchange Ratio

3.2.4 Statistics

Two kinds of statistical designs were used to assess the data:
A paired test to assess pre. vs post exercise effects on physiological and biochemical parameters and unpaired test to compare differences between the untrained and trained cohort.
Paired tests: Individual parameters characterising metabolites with exercise were assessed with a repeated ANOVA (STATISTICA) and a post-hoc test of Fisher. This latter test is also a T-test.

Due to the multi-factorial nature of the metabolome data general effects on the metabolite peaks were assessed with a repeated ANOVA followed up with significance analysis of microarrays (SAM). This was necessary to account for the strong influence of the multiple tests on a type I error. Unpaired T-Tests were applied to assess differences in anatomical and physiological values between the trained vs. untrained cohort (MS-Excel, Kildare, IRL). To reiterate errors of multiple comparisons were taken care of with SAM for microarray and metabolomic data which controls false discorvery rate.

3.3. Results

Endurance trained and untrained individuals were of similar age, height and body mass. Although there was no significant difference between trained and untrained individuals in BMI, the DEXA scan revealed that endurance trained participants tended to have lower body fat percentage (P=0.06) (Table 11). The MRI images of the thigh muscles shown in Figure 35 for trained (left) and untrained (right) individuals revealed that endurance trained participants tended to have higher muscle volume (Table 12 and 14). When each of the four quadriceps muscles was analysed only the *vastus intermedius* muscle volume was significantly different between the trained and untrained participants (Table 14, P=0.02). The percentage of Type I

muscle fibre and capillary number did not differ significantly between the trained and untrained participants (Table 13).

Endurance trained participants had significantly higher aerobic fitness, as indicated by higher VO_2max, higher local muscle VO_2peak and higher power output during both two-legged and single-legged exercise (Table 11). There was no significant difference

Table 11. Anthropometry of the trained and untrained cohort.

	Training Status				
	Trained (n=11)		Untrained (n = 9)		P
Age (years)	27.3	(19:38)	24.8	(19:34)	0.39
Body Mass (Kg)	79.6	(69:99)	74.8	(60:99)	0.32
Height (M)	1.83	(1.77:1.94)	1.79	(1.37:1.91)	0.22
BMI (Kg/M^2)	23.7	(21.0:29.6)	23.3	(18.5:30.9)	0.78
Body Fat (%)	14.8	(6.1:28.0)	21.0	(10.7:32.1)	**0.06**

The data is displayed as mean (range), Body Fat (%) demonstrated a trend for being different between the trained and untrained cohort, as highlighted by underlining

between trained and untrained individuals in RER at any point during the two-leg cycling maximal oxygen uptake test, with the exception of the final 15 seconds, where the untrained participants had a higher RER (1.04 vs. 1.12; P=0.02; Figure 36).

Table 12. Parameters of aerobic fitness in the trained and untrained cohorts.

	Training Status		
	Trained (n=11)	**Untrained (n = 9)**	**P**
VO_2max (ml/min/Kg): 2-leg	60.3 (50.1:70.1)	48.2 (43.2:57.6)	**0.0001**
VO_2peak (ml/min/Kg): 1-leg	48.4 (42.1:56.0)	42.3 (34.2:48.9)	**0.01**
VO_2max (ml/min): 2-leg	4774 (3848:5319)	3564 (3115:4639)	**0.00005**
VO_2peak (ml/min): 1-leg	3845 (3148:4719)	3128 (2937:4093)	**0.00003**
Pmax (W): 2-leg	369 (290:450)	267 (230:340)	**0.00005**
Pmax (W): 1-leg	234 (190:275)	168 (140:220)	**0.007**
Pmax (W/Kg): 2-leg	4.64 (3.92:5.42)	3.58 (3.31:4.00)	**0.00001**
Pmax (W/Kg): 1-leg	2.95 (2.57:3.41)	2.26 (2.00:2.75)	**0.00002**

The data is displayed as mean (range). All parameters of aerobic fitness are significantly different between the untrained and trained cohorts, as underlined

Table 13. Mean and extreme values for three muscle parameters in the two cohorts.

	Training Status		
	Trained (n=11)	**Untrained (n = 9)**	**P**
Capillary density (mm^{-2})	300 (246:345)	283 (207:352)	0.35
Fibre type I (%)*	44.5 (22.0:63.0)	37.9 (27.2:52.6)	0.25
Quadriceps volume (cm^3)	2348 (1802:2949)	1957 (1419:2468)	**0.04**

The data is displayed as mean (range). *n=8, untrained. Only quadriceps volume is significantly different between cohorts, as underlined

Table 14. Quadriceps mucle volume of untrained and trained participants.

Untrained

Quadriceps Volume (mm^3)				Total Volume (mm^3)	
RF	**VL**	**VM**	**VI**		
225	467	352	375	1419	
285	530	404	447	1666	
258	643	548	521	1970	
326	569	503	695	2093	
320	740	530	693	2283	
370	625	616	679	2290	
279	570	400	502	1751	
237	521	401	513	1672	
377	828	561	702	2468	
297.4	**610.3**	**479.4**	**569.7**	**1956.9**	***Mean***
18.21	**37.97**	**30.62**	**41.41**	**117.54**	***Se***

Trained

Quadriceps Volume (mm^3)				Total Volume (mm^3)	
RF	**VL**	**VM**	**VI**		
316	681	542	594	2133	
326	670	494	625	2115	
287	671	571	678	2207	
386	817	700	765	2668	
292	523	402	585	1802	
358	748	801	1029	2936	
530	888	649	852	2919	
270	619	554	710	2153	
332	591	447	546	1916	
406	894	722	927	2949	
290	525	453	761	2029	
344.8	**693.4**	**575.9**	**733.8**	**2347.9**	***Mean***
22.56	**39.43**	**38.50**	**45.99**	**130.74**	***Se***
0.13	0.15	0.07	0.02	**0.04**	***P***

RF: rectus fermoras, VL: vastus lateralis, VM: vastus medialis, VI: vastus intermedius. Underlined denotes significantly different between trained and untrained (unpaired T-test).

Trained participant, with a body fat percentage of ~ 6%

Untrained participant, with a body fat percentage of ~ 32%

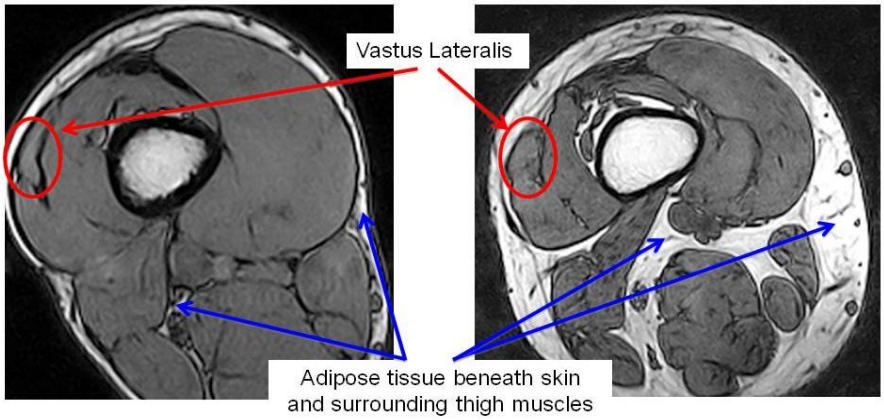

Figure 35. Local body fat (surrounding quadriceps muscles) differences between a trained and untrained participant

Both images are taken at approximately the same point along the quadricep muscle – approximately at the location where the *vastus lateralis* muscle starts to appear (its origin) as highlighted by the red circle. This is figure is displayed for illustrative purposes to highlight the physiological (clear) differences between someone who regularly exercises and someon who does not

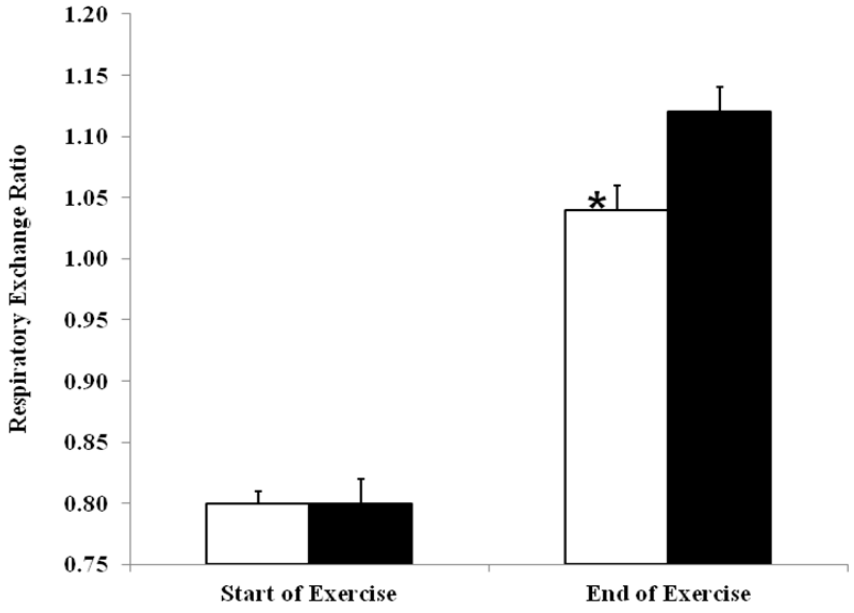

Figure 36. RER at the start and end of a single bout of two-legged aerobic exercise

*P=0.02 vs untrained. RER values are means of 15 seconds taken at the: start of exercise (2 mins), and at the end (at maximal volition, just before cessation of exercise bout – 15 seconds left). Data are expressed as mean ± SEM, trained n=10, untrained n=8. ■ Untrained □ Trained. Both trained and untrained cohorts had an RER at the end of exercise, which was significantly different, P<0.0000001

Table 15. Serum metabolites pre- and post-exercise (two-legged) in trained and untrained cohorts.

					T	**UT**	**Pre-Ex**	**Post-Ex**
	T		**UT**		**Pre vs. Post**		**T vs. UT**	
(mmol/l)	**Pre-Exercise**	**Post-Exercise**	**Pre-Exercise**	**Post-Exercise**	*P*	*P*	*P*	*P*
Glucose	3.92 (3.2:4.9)	4.92 (3.5:7.3)	4.1 (3.3:5.4)	4.56 (3.0:6.9)	**0.03**	0.43	0.54	0.57
Total Chol	3.94 (3.27:5.37)	4.11 (3.38:6.02)	3.58 (3.07:4.19)	3.53 (3.01:4.19)	0.59	0.82	0.16	0.09
HDL	1.00 (0.70:175)	1.33 (0.81:4.0)	1.00 (0.65:1.61)	1.04 (0.65:1.71)	0.31	0.24	0.97	0.43
LDL	2.82 (2.24:3.89)	2.67 (2.06:3.12)	2.39 (1.88:2.83)	2.29 (1.83:2.76)	0.47	0.53	**0.04**	0.06
TAG	0.61 (0.57:0.80)	0.61 (0.57:0.75)	0.95 (0.57:2.72)	0.99 (0.57:2.65)	0.89	0.90	0.12	0.21
Ketones	0.58 (0.32:0.89)	0.48 (0.34:0.58)	0.55 (0.19:0.90)	0.49 (0.28:0.72)	0.38	0.33	0.76	0.91

The data is displayed as mean (range). Glucose levels pre vs. post in trained and LDL levels Pre-Ex in trained vs. untrained were significantly different as, as underlined.

T: Trained, UT: Untrained, Pre-Ex: Pre-exercise, Post-Ex:Post-Exercise, Total Chol: Total Cholesterol, HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein, TAG: Triacylglyceride. n=8 UT cohort (for all measures), n=10 Glucose and HDL, n=9 Total Chol, n=8 Ketones, n=6 LDL and TAG.

Blood samples were taken at the beginning and the end of the two-legged cycling VO_2max test and were measured for serum concentrations of lipids, ketones and glucose metabolites. Serum LDL was significantly higher in trained participants in the fasted state at rest and following the two-legged VO_2max test (P=0.04 and P=0.06, respectively). None of the other measured metabolites differed between trained and untrained participants in the rested state. From the measured metabolites, only glucose concentration showed a significant change after exercise in the trained individuals. Untrained participants showed no exercise-induced change in any of the measured metabolites (Table 15).

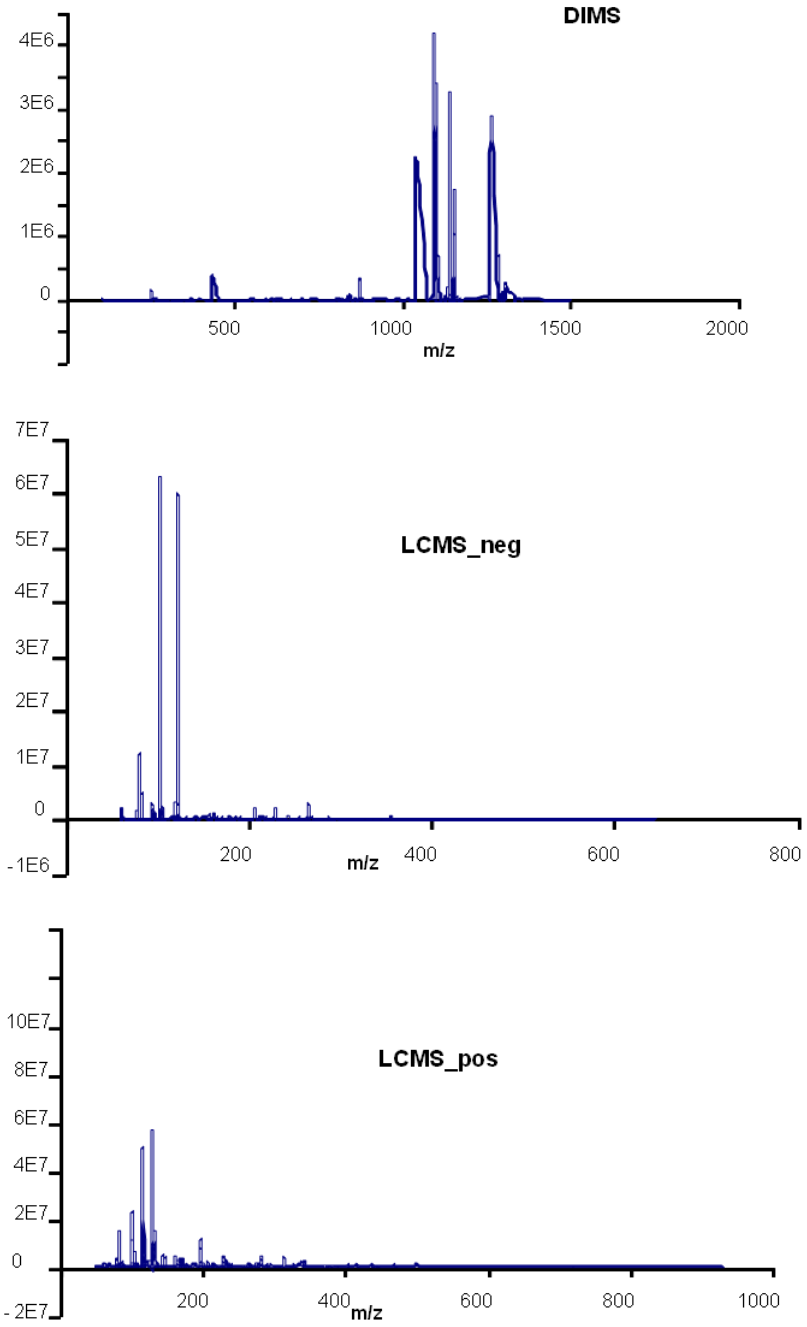

Figure 37. Metabolite reference profiles (of three different classes)

Line graph visualizing the reference profiles for the peaks identified with mass spectroscopy in the polar and non-polar fraction of *vastus lateralis* muscle prior to endurance exercise. Please note the units are dimensionless – reflect intensity of signal (the signal represents the signal intensity of the different ions of a given m/z ratio. It is related to Volts but this is not absolute and depends on instrument calibration. To take care of this signals were related to reference profiles, which are shown in these graphs – they reflect the median of 'signal intensity' for each m/z data point. DIMS, direct injection mass spectroscopy (MS) (polar fraction); LCMS_ESI+, liquid chromatography MS in positive electrospray ionisation mode (LCMS_pos) (non-polar fraction); LCMS_ESI-, liquid chromatography MS in negative electrospray ionisation mode (LCMS_neg) (non-polar fraction).

There was no significant difference between trained and untrained participants in the non-polar (Figure 38) or the polar (Figure 39) metabolites in the rested and fasted state. Both groups showed significant increases in the non-polar and the polar metabolites following the single-leg exercise bout, but the increase that occurred, in non-polar metabolites, in the endurance trained individuals was significantly higher than the increase seen in the untrained participants (P=0.0004; Figure 38). The opposite was seen in the polar metabolites, where untrained individuals had significantly higher increases in polar (positive and negative) metabolites than endurance trained individuals (P<0.00001; Figure 39)

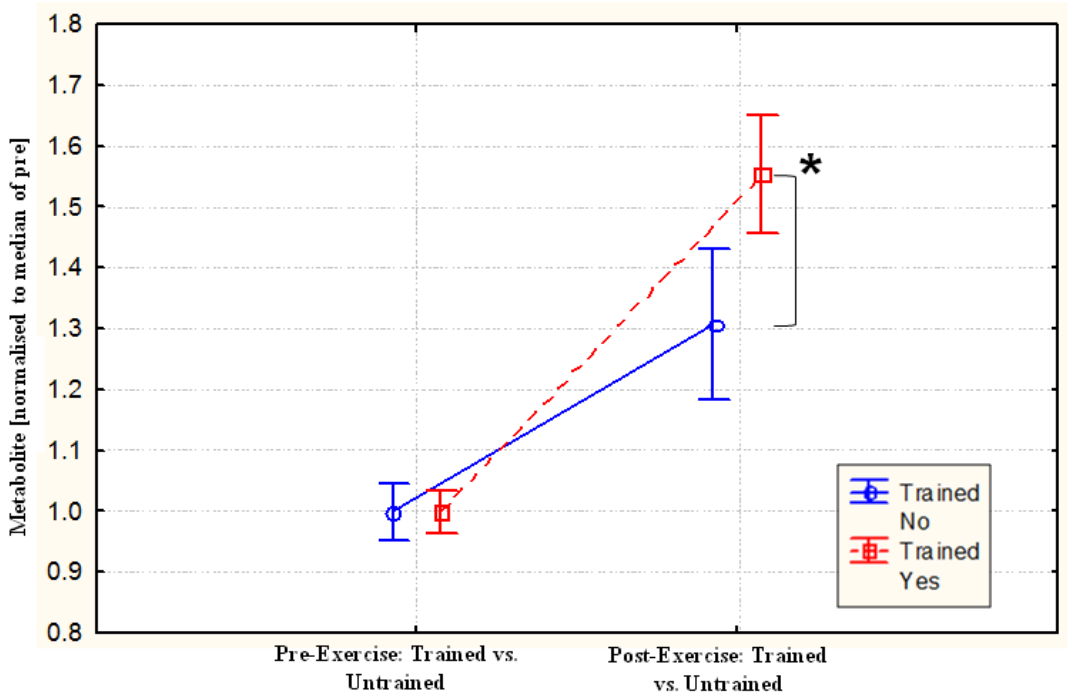

Figure 38. Interaction effect of an acute exercise bout and training state on non-polar metabolites

*P=0.0004, repeated ANOVA mean fold change post-exercise: trained vs. untrained. Vertical bars denote 0.95 confidence intervals. Normalised data to pre-exercise (T & UT) = 1.0

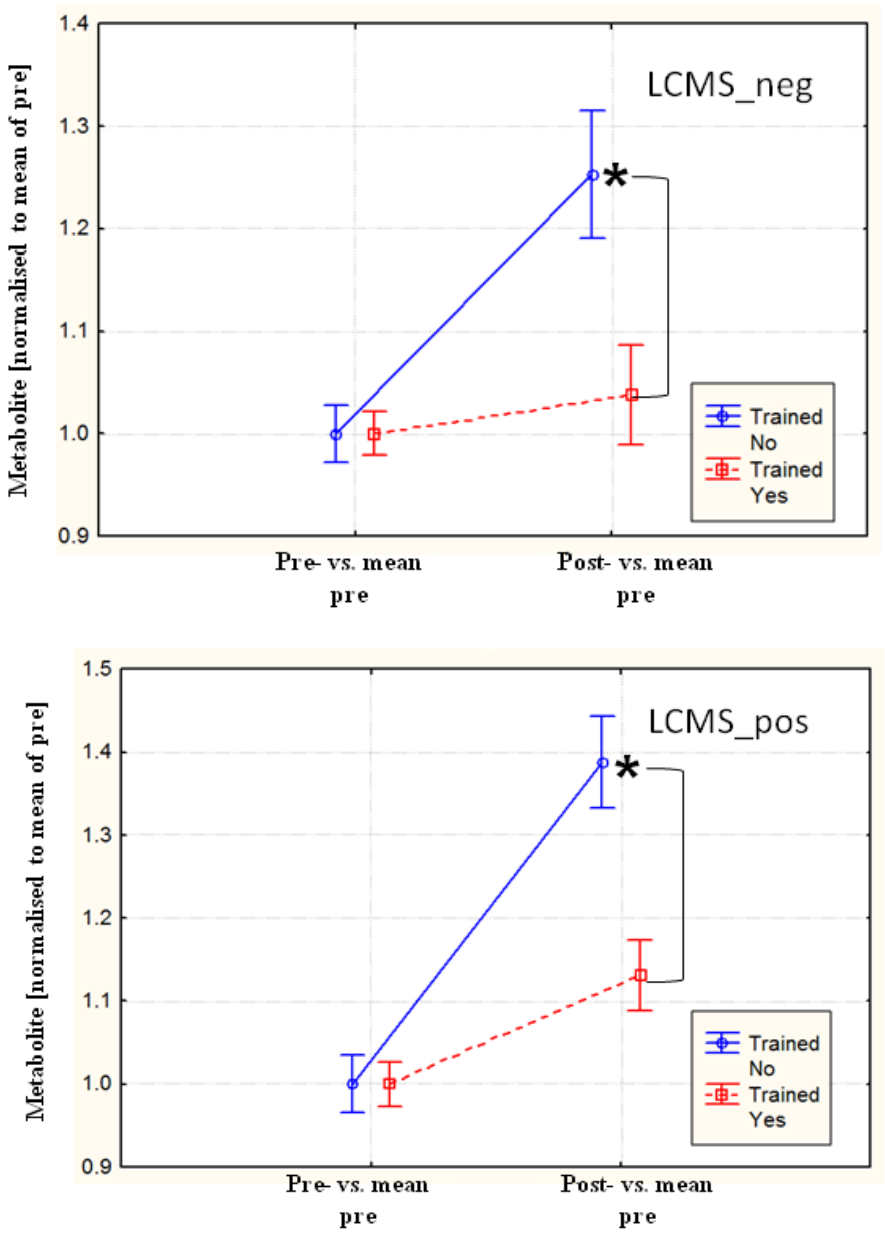

Figure 39. Interaction effect of exercise and training state on polar metabolites

*P<0.00001, repeated ANOVA mean fold change post-exercise: trained vs. untrained. Vertical bars denote 0.95 confidence intervals. . Normalised data to pre-exercise (T & UT) = 1.0

Identification of the metabolites that caused the peak in the results trace: The specific polar metabolites that caused the peak in the results trace have not yet been identified, but preliminary studies have been conducted to identify the non-polar metabolites. SAM identified six metabolite peaks in the non-polar fraction of muscle extracts that were significantly different between trained and untrained participants in the rested, fasted state (Table 16). In the untrained participants, there were four non-polar metabolites that increased after single-leg endurance exercise (Table 17).

In trained participants there were 76 metabolites increased following the bout of single-legged endurance exercise (Table 17). The results are shown in tables 16-18 with the altered DIMS peaks and their putative identification. Metabolites are grouped (putatively) by main- and then sub-class (in alphabetical order).

Table 16. Metabolite difference, in skeletal muscle, pre acute exercise bout in trained versus untrained participants.

Peak ID	Fold Change (median)	q-value (%)	True Mass	Multiple Hits	Common name	Putative ID (sub class)	Putative ID (main class)
S1154.4	4.1	0.0	1155.32	No	None	Anthocyanidins	Flavonoids
S1342.1	1.8	0.0	1343.35	No	None		
S1360.1	2.1	0.0	1359.81	No	None	GalNAcβ1-3Galα1-4Galβ1-4Glc-	Neutral glycosphingolipids
S704.5	2.6	0.0	705.49	Yes	PS(O-16:0/15:1(9Z))	1-alkyl,2-acylglycerophosphoserines	Glycerophosphoserines
S817.8	2.1	0.0	818.74	Yes	TG(16:0/16:1(9Z)/17:0)[iso6]	Triradylglycerols	Triradylglycerols
S993.2	1.5	0.0	994.26	No	Quercetin 3-(6'''-sinapylsophorotrioside)	Flavones and Flavonols	Flavonoids

q-value: analogue of the P value, used by SAM to correct for multiple testing (see Storey 2003)

Table 17. Changes to skeletal muscle metabolites following an acute exercise bout in previously untrained participants.

Peak ID	Fold Change (median)	q-value (%)	True Mass	Multiple Hits	Common name	Putative ID (sub class)	Putative ID (main class)
S436.3	1.22	0	437.33	Yes	arachidonyl amine	N-acyl amines	Fatty amides
S331.3	1.73	0	332.27	Yes	Adrenic Acid	Unsaturated fatty acids	Fatty Acids and Conjugates
S310.3	1.15	0	311.28	2	(4E,8E,9Me-d19:2)sphingosine	Sphingoid base analogs	Sphingoid bases
S310.2	1.16	0	311.28	2			

q-value: analogue of the P value, used by SAM to correct for multiple testing (see Storey 2003)

Table 18. Metabolites increased, in skeletal muscle, following an acute exercise bout in trained participants.

Peak ID	Fold Change (median)	q-value (%)	True Mass	Multiple Hits	Common name	Putative ID (sub class)	Putative ID (main class)
S1126.3	1.3	0.00	1128.8	2	None	Gangliosides	Acidic glycosphingolipids
S1264.1	0.8	4.91	1264.84	No	None		
S1451	2.5	4.91	1452.91	No	None		
S604.5	0.9	4.91	605.57	Yes	Cer(d18:2/21:0)	N-acylsphingosines	Ceramides
S620.6	0.9	0.00	621.54	No	DG(18:2(9Z,12Z)/0:0/18:2(9Z,12Z)) (d5)		
S563.5	1.0	4.91	564.48	No	DG(16:1(9Z)/16:1(9Z)/0:0)	Diacylglycerols	Diradylglycerols
S667.6	3.6	4.91	668.54	Yes	DG(18:1(9Z)/22:5(7Z,10Z,13Z,16Z,19Z)/0:0)[iso2]		
S689.5	1.7	0.00	690.52	Yes	DG(20:5(5Z,8Z,11Z,14Z,17Z)/22:4(7Z,10Z,13Z,16Z)/0:0)[iso2]		
S1299.4	1.0	4.91	1301.32	2	Isocaldarchaeol	Di-glycerol tetraethers	
S575.5	4.7	4.91	576.48	No	DG(16:1(9Z)/17:2(9Z,12Z)/0:0)[iso2]	Diradylglycerols	
S323.2	1.0	2.13	324.19	2	Dinor-PGE2	Prostaglandins	Eicosanoids
S129.1	1.0	1.22	130.1	Yes	a-ethyl valeric acid	Branched fatty acids	Fatty Acids and Conjugates
S309.2	6.5	4.91	310.21	Yes	None	Hydroperoxy fatty acids	
S535.5	192921.2	0.00	536.55	Yes	Hexatriacontylic acid	Straight chain fatty acids	
S525.5	2.8	4.91	526.5	No	36:5(21Z,24Z,27Z,30Z,33Z)	Unsaturated fatty acids	
S253.2	1.9	0.00	254.22	Yes	cis-10-palmitoleic acid		
S331.3	2.7	0.00	332.27	Yes	DHA (d5)		
S185.2	1.2	1.22	186.2	No	None	None	Fatty alcohols
S255.4	1.1	4.91	256.28	Yes	None	None	
S312.3	3.3	0.00	313.3	No	Margaroyl-EA	Endocannabinoids	Fatty amides
S212.1	0.9	0.00	213.1	2	None	Fatty acyl homoserine lactones	

q-value: analogue of the P value, used by SAM to correct for multiple testing (see Storey 2003)

Table 18 continued. Metabolites increased, in skeletal muscle, following an acute exercise bout in trained participants.

Peak ID	Fold Change (median)	q-value (%)	True Mass	Multiple Hits	Common name	Putative ID (sub class)	Putative ID (main class)
S1260	0.9	2.13	1259.33	No	None	Anthocyanidins	Flavonoids
S1140.9	1.1	4.91	1141.3	No	None		
S1141.3	0.9	4.91	1141.3	No	None		
S1288	1.3	4.91	1289.3	No	None		
S1150.8	1.0	4.91	1151.29	No	None		
S484.4	1.0	1.22	484.36	Yes	Epigallocatechin 3-O-(3,5-di-O-methylgallate)	C21 steroids (gluco/mineralocorticoids, progestogins) and derivatives	
S281	1.9	2.13	282.05	Yes	9-O-Methylcoumestrol	Coumestan flavonoids	
S417.2	1.3	1.22	418.14	No	Cyclomorusin	Flavones and Flavonols	
S594.9	6.2	2.13	596.14	Yes	Quercetin 3-lathyroside		
S268.2	0.6	4.91	270.05	Yes	Genistein	Isoflavonoids	
S685.5	128432.8	2.13	685.5	Yes	PE(P-16:0/17:2(9Z,12Z))	Diacylglycerophosphates	Glycerophosphates
S814.5	0.9	4.91	815.58	Yes	None	1-acyl,2-alkylglycerophosphocholines	Glycerophosphocholines
S810.6	1.1	4.91	811.61	Yes	PC(18:0/20:3(5Z,11Z,14Z))	Diacylglycerophosphocholines	
S896.7	1.4	4.91	897.72	No	PC(22:1(13E)/22:1(13E))		
S834.6	0.7	1.22	835.61	Yes	PC(18:0/22:5(4Z,7Z,10Z,13Z,16Z))		
S812.5	2.4	1.22	813.53	Yes	PE(20:3(8Z,11Z,14Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Diacylglycerophospho-ethanolamines	Glycerophosphoethanolamines
S808.5	1.0	0.00	809.5	2			
S810.5	1.6	0.00	811.52	Yes	PE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/20:4(5Z,8Z,11Z,14Z))		
S805.6	1.2	0.00	806.6	No	PG(17:0/21:0)	Diacylglycerophosphoglycerols	Glycerophosphoglycerols
S827.5	0.5	2.13	828.59	Yes	PG(20:0/20:3(8Z,11Z,14Z))		
S843.6	1.1	4.91	844.62	Yes	PG(22:2(13Z,16Z)/19:0)		
S759.5	0.7	0.00	760.5	Yes	PA(19:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))		
S717.5	1.0	0.00	718.51	Yes	PG(P-16:0/17:1(9Z))	1-alkyl,2-acylglycerophosphoglycerols	

q-value: analogue of the P value, used by SAM to correct for multiple testing (see Storey 2003)

Table 18 continued. Metabolites increased, in skeletal muscle, following an acute exercise bout in trained participants.

Peak ID	Fold Change (median)	q-value (%)	True Mass	Multiple Hits	Common name	Putative ID (sub class)	Putative ID (main class)
S809.5	1.3	0.00	810.53	Yes	PI(16:0/16:0)	Diacylglycerophosphoinositols	Glycerophosphoinositols
S829.5	0.8	4.91	830.49	Yes	PI(18:3(6Z,9Z,12Z)/16:1(9Z))		
S875.5	1.2	4.91	876.64	No	None		
S825.5	0.7	1.22	826.46	Yes	PI(14:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z))		
S873.5	2.8	4.91	874.56	Yes	PI(15:1(9Z)/22:2(13Z,16Z))		
S802.6	1.0	0.00	802.6	Yes	PG(P-20:0/19:1(9Z))	1-alkyl,2-acylglycerophosphoserines	Glycerophosphoserines
S828.6	1.4	0.00	829.62	Yes	PS(O-18:0/22:2(13Z,16Z))		
S826.6	1.8	1.22	827.6	Yes	PS(O-20:0/20:3(8Z,11Z,14Z))		
S848.6	25856.0	2.13	849.59	No	PS(O-20:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))		
S824.5	1.1	1.22	825.53	Yes	PC(18:4(6Z,9Z,12Z,15Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Diacylglycerophosphocholines	
S768.5	159282.4	0.00	769.49	Yes	PS(13:0/22:4(7Z,10Z,13Z,16Z))	Diacylglycerophosphoserines	
S854.6	0.6	4.91	855.6	Yes	PS(19:1(9Z)/22:2(13Z,16Z))		
S840.6	1.1	2.13	841.58	Yes	PS(18:1(9Z)/22:2(13Z,16Z))		
S724.4	5.0	4.91	725.43	Yes	PS(18:4(6Z,9Z,12Z,15Z)/14:1(9Z))		
S866.6	1.8	2.13	867.6	Yes	PS(20:3(8Z,11Z,14Z)/22:1(11Z))		
S518.2	2.0	2.13	518.19	No	Gossypol	Monoacylglycerophosphoserines	
S846.8	1.2	4.91	847.67	Yes	PS(O-20:0/21:0)	1-alkyl,2-acylglycerophosphoserines	
S790.5	0.1	1.22	791.5	Yes	SQDG(16:0/16:1(9Z))	Glycosyldiacylglycerols	Glycosyldiradylglycerols

q-value: analogue of the P value, used by SAM to correct for multiple testing (see Storey 2003)

Table 18. continued. Metabolites increased, in skeletal muscle, following an actue exercise bout in trained participants.

Peak ID	Fold Change (median)	q-value (%)	True Mass	Multiple Hits	Common name	Putative ID (sub class)	Putative ID (main class)
S573.5	1.0	0.00	574.47	No	Dihydroxyneurosporene/ OH-Chloroxanthin	C40 isoprenoids	Isoprenoids
S811.6	1.2	1.22	812.6	No	PG(O-18:0/22:4(7Z,10Z,13Z,16Z))		
S811.5	1.2	2.13	812.46	Yes	bayogenin 3-O-cellobioside	C30 isoprenoids	
S1332.1	1.0	1.22	1331.78	No	None	GalNAcß1-3Gala1-4Galß1-4Glc- (Globo series)	Neutral glycosphingolipids
S1359.1	1.1	1.22	1359.81	No	None		
S1446.9	1.0	4.91	1447.43	No	Vancomycin	None	Non-ribosomal peptide/polyketide hybrids
S267.2	0.6	4.91	268.2	2	None	12-oxophytodienoic acid metabolites	Octadecanoids
S310.2	6.0	4.91	311.28	2	(4E,8E,9Me-d19:2)sphingosine	Sphingoid base analogs	Sphingoid bases
S310.3	362866.0	4.91	312.28	2	(4E,8E,9Me-d19:2)sphingosine		
S328.2	181096.0	0.00	329.29	Yes	Penaresidin A		
S692.6	77797.4	0.00	693.57	No	Cholesteryl nitrolinoleate	Cholesteryl esters	Sterols
S847.8	0.9	2.13	848.78	No	TG(17:0/17:0/17:0)	Triradylglycerols	Triradylglycerols
S833.8	0.8	4.91	834.77	2	TG(16:0/16:0/18:0)		
S845.8	1.0	2.13	846.77	Yes	TG(17:0/17:0/17:1(9Z))[iso3]		

q-value: analogue of the P value, used by SAM to correct for multiple testing (see Storey 2003)

3.4. Discussion

The main purpose of this study was to examine the response of lipid species to an acute bout of endurance-type exercise and to compare the responses between sedentary and endurance trained individuals. The major novel findings were that trained and untrained individuals both showed significant exercise-related increases in non-polar lipid metabolites within the musculature, but the observed increases were much greater in trained individuals. There were also significant differences in polar metabolite responses following an acute exercise bout, but no preliminary identification has occurred on these to date (further elaboration is not possible as no further analysis was performed due to no more financial or technical resources remaining.

Overall results reveal the complex substrate and energetic response to exercise and suggest that through exercise training it is possible to up regulate lipid metabolic responses. It should be noted that the non-polar metabolites have only been tentatively identified, therefore the following text should be read with this firmly in mind.

The endurance-trained individuals in this study had higher aerobic capacity compared with the untrained participants and had lower body fat and larger leg muscles, which are all probable adaptations to their exercise training regimens. During the two-legged exercise bout, we did not observe differences between trained and untrained participants in the RER at submaximal exercise intensities, but it is pointed out that the ramped incremental protocol utilised in the present study is not appropriate to examine fuel utilisation (reflected in the steady-state RER) because participants never reach a steady state. Thus, although not demonstrated in this study, it is widely known that endurance trained individuals have higher rates of fat oxidation and mitochondrial density (Gollnick *et al.*, 1973; Hoppeler *et al.*, 1985). It is possible that the marked increase in 76 lipid species seen in the musculature of trained participants as well as higher circulating LDL following exercise is related to an enhanced localised fatty acid oxidation and overall lipid metabolism.

The lipid species have been tentatively identified (putative ID - main class, sub class). Many were glycerophospholipids – Table 18. These are primarily membrane components but they can also produce “lipid mediators that not only transduce signals from the cell surface to interior, but also modulate intracellular metabolism, ion

transport, and gene expression" (Mathews *et al.*, 2000; Farooqui, 2009). These same compounds are involved in fatty acid metabolism, especially TAG and cholesterol transport (Mathews *et al.*, 2000). Interestingly the physiologically potent compound Platelet Activating Factor (PAF-1) has the same structure as the compound 1-alkyl,2-acylglycerophosphocholines Ttable 18) (Mathews *et al.*, 2000). This compound has many important physiological effects including stimulation of blood platelet aggregation, reduction of blood pressure and stimulation of glycogenolysis (Mathews *et al.*, 2000). Diradylglycerols were also significantly increased in trained participants following a single exercise bout. They are thought to stimulate phospholipase A2 (itself a key enzyme involved in the inflammatory/signalling pathway and importantly involved in hydrolysis of glycerphospholipids (Mallat *et al.*, 2010)), and is a key intracellular messenger involved in the mediation of agonist-induced vascular smooth muscle cell contraction (Hui *et al.*, 1992). Eicosanoids were also increased after training. They are important signalling molecules, controlling inflammation and are derived from DAG's and phospholipids. Prostaglandins, a subclass of eicosanoids, have been shown to be involved in vaso-constriction/-dilatation and angiogenesis (Lord *et al.*, 2007). The unsaturated fatty acid docosahexaenoic acid (DHA) up-regulates fatty acid oxidation in skeletal muscle (and increases total glycogen storage) (Clarke, 2001), and is an activating ligand for many transcriptional factors controlling beta-oxidation (Clarke, 2001). DAG, TAG, fatty acids, cholesterol esters, and gangliosides are further examples of tentative lipid species, which are important in lipid metabolism. These data suggest that trained participants demonstrate a clear up-regulation of lipid species, which supports the general view of a greater reliance on lipid metabolism in conditioned working muscle.

Our observations of altered skeletal muscle lipid species following exercise, reported above, were made after the participants completed a bout of intense single-legged endurance exercise. Although two-legged exercise is more conventional and better reflects usual physical activities in daily life, it has been shown that the leg muscles of different people can receive different exercise intensity during moderate intensity conventional endurance exercise (McPhee, 2009). Single-leg exercise was therefore chosen to ensure that the exercise stimulus received by the working muscles was similar in all participants, irrespective of differences in training status, stature or other physiological variables such as cardiac output. The single-leg exercise bout was

set at 60% Pmax and continued for 20 minutes. At this exercise intensity it is expected that fat oxidation would be at or close to maximum, but glucose metabolism would also be concomitantly high. The RER during single-leg exercise of both trained and untrained subjects reached a steady state at around 0.89, indicating that a combination of fat and glucose was being utilised. But, it is pointed out that the RER during single-leg work does not provide a true representation of the metabolism of the exercising leg because the gas exchange was measured at the pulmonary level where it had been diluted and influenced by metabolism in other parts of the body.

To the author's knowledge this study is the first time metabolites have been (tentatively) identified in skeletal muscle in untrained, trained; at rest and following a single-legged bout of endurance exercise. Previous work investigated serum metabolites (Yan *et al.*, 2009; Lehmann *et al.*, 2010; Lewis *et al.*, 2010), their profile at rest, post exercise and in trained individuals. However, our results here, measured from the non-polar fraction of skeletal muscle do suggest that there may be important metabolites related to energy metabolism (especially lipid), which are differently regulated in trained populations. Interestingly, these do not belong to the conventional metabolites/pathways. This is in agreement with Yan et al. (2009), who found no differences in "normal" biochemical parameters associated with training.

It remains a possibility that a different metabolic response of lipid species could be observed following two-legged endurance exercise and/or exercise of longer duration, where it is known that fat oxidation contributes a relatively greater fraction of the overall energy utilisation.

3.5. Conclusion

Endurance trained and untrained individuals did not differ significantly for their skeletal muscle lipid species when in a fasted, rested state. However, following a bout of high-intensity single-leg endurance exercise, the trained individuals' upregulated 76 lipid species whereas the untrained individuals upregulated only 4. These results suggest that trained individuals display a much greater ability to up-regulate lipid pathways during endurance exercise. A major theme in the affected metabolite classes concern lipid mediators involved in signal transduction as initiated

in the plasma membrane (i.e. glycerophospholipids). It also appears that the affected pathways are not part of metabolic substrate pathway that fuel contraction but rather plasma membrane associated. This may indicate signalling?

Chapter 4

Does the ACE I/Dp Genotype Modify Plasticity of Skeletal Muscle in Untrained and Trained British Citizens?

4.1. Introduction: molecular and metabolite responses to muscle whole-body work

Exercise phenotypes and the implication of muscle plasticity to adjustments with training. New UK physical activity guidelines were produced in July 2011 by the four home countries' Chief Medical Officers: Start Active, Stay Active (CMO, 2011). This document laid out key recommendations that highlighted the importance of regular exercise for health: at least 150 minutes per week, mixed modalities of exercise, e.g. endurance and resistance, and stressed the importance of intensity – specifically stating *vigorous* type exercise. Finally there was a recommendation that sedentary type behaviour (watching TV, sitting, reading etc) should also be reduced, and that this in itself is a health risk, even in those meeting physical activity recommendations. The link between sedentary behaviour being detrimental to long-term health is not new to those in the field of exercise physiology and health (Booth *et al.*, 2002; Hu *et al.*, 2003; Lees & Booth, 2004) (see section 1.1.3, Table 3). But, this important point has been now been recognised in guidelines intended for the general public.

Regular exercise has many benefits (see section 1.1.3), of which there are three basic exercise or training patterns that can be followed: endurance, strength or a combination of these (as recommended by the new UK physical activity guidelines: CMO, 2011). The type of exercise has a profound effect on physiological adaptations, especially adaptations of muscle (see Figure 2).

Endurance type training is associated with increases in whole-body oxygen uptake capacity and aerobic fitness through local and systemic related metabolic and local changes especially processes of energy metabolism. With the most notable adaptations being: increases in mitochondrial (and related enzymes) and capillary density (Gollnick *et al.*, 1972; Costill *et al.*, 1976; Spurway & Wackerhage, 2006) – occurring preferentially in slow oxidative fibres (Rossiter *et al.*, 2005) – plasma volume and myoglobin content (Astrand, 2003; Spurway & Wackerhage, 2006), TCA enzymes (Jones, 2004), heart size (left ventricular hypertrophy) and stroke volume (Gledhill *et al.*, 1994; Moore & Palmer, 1999; Astrand, 2003).

Strength type training is associated with stimulation of signalling pathways specific to the hypertrophic response (Spiering *et al.*, 2008) and subsequent protein synthesis

(providing adequate nutrition) leading to increased fibre size (hypertrophy) (Astrand, 2003; Jones, 2004; Atherton *et al.*, 2005; Spurway & Wackerhage, 2006). Although it is important to note that physiological and structural adaptations are exercise specific, it is possible to observe both increases in capillarity and mitochondrial density in highly glycolytic muscle – if the exercise type mainly stimulates these fibres, such as interval sprint training (Gute *et al.*, 1994; Glowacki *et al.*, 2004). Concurrent exercise training (endurance and strength) was first thought to interfere, where strength training gains were prevented by endurance training (Hickson, 1980). However, since then studies have both supported (Craig, 1991; Hennessy, 1994; Bell *et al.*, 2000) and refuted this theory (Sale *et al.*, 1990; Wood *et al.*, 2001; McCarthy *et al.*, 2002).

From chapter two we provided evidence that adjustments in skeletal muscle, recruited during exercise, play an important role for fine-tuning in metabolic fitness with endurance training. In this chapter we will provide evidence that a specific genetic polymorphism modifies the implicated biochemical process in skeletal muscle.

The rest of this chapter will focus on the related effects of endurance training/exercise.

Gene expression reflects remodelling of muscle: Molecular biology has produced a highly resolved picture on the sequential time course and breadth of adaptation to external stressors. With the advent of technologies like microarrays the expression of hundreds (if not thousands) of different genes have been studied in skeletal muscle (Cameron-Smith, 2002; Keller *et al.*, 2011). Sections 1.1.3, 1.1.4 and 1.1.5 provide further detail on genes/gene expression and exercise. In addition some of the more studied transcript level adjustments, during recovery from endurance exercise, are briefly mentioned here, especially those involved in energy metabolism and vascular remodelling.

ATP provides the energy for muscle contraction, therefore anything that affects its availability or levels will have profound effects. It is the splitting of the high energy phosphate bonds to liberate Pi from the ATP molecule that provides the energy to drive muscle contraction. This then leaves ADP, which can be further broken down to AMP. As rates of ATP turnover are increased with increasing intensity of exercise, so the concentration of the intermediates ADP and AMP are also increased. AMP can be thought of as the cells energy sensor, involved in stimulating the activity of a key

phosphorylation enzyme: 5'adenosine monophosphate activated protein kinase (AMPK) (Hardie & Sakamoto, 2006). AMPK plays a key role in activation of fatty acid metabolism (Winder *et al.*, 2000), especially involving the activation of a key mitochondrial biogenesis regulator, PPAR-γ coactivator-1α (PGC-1α) (Wu *et al.*, 1999), but also stimulates the expression of GLUT-4 receptors and the translocation of GLUT-4 to the cell membrane to enhance the take-up of glucose into the cell (Holmes *et al.*, 2005). AMPK is not the only signalling molecule activated as a consequence of muscle contraction. It is now appreciated that muscle contraction produces very many stimuli that have been shown to activate a variety of signalling processes, from substrate intermediates, to calcium, hypoxia, mechanical stretch-activated proteins, CO_2 and so on (Booth, 1996; Fluck & Hoppeler, 2003). Other genes whose expression levels are of interest following endurance exercise (some well studied, some less so) include: lipoprotein lipase (LPL – lipid metabolism, (Pilegaard *et al.*, 2000)), vascular endothelial growth factor (VEFG – angiogenesis (Richardson *et al.*, 1999)), cytochrome c oxidase subunit I/IV (COX-I/IV – components of the respiratory chain in mitochondria, (Hood, 2001)), mitochondrial transcription factor A (Tfam – involved in mitochondrial biogenesis, (Hood, 2001)), hypoxia inducible factor 1 (HIF-1, transcription factor responding to low oxygen levels, and which regulates the expression of other genes involved in energy metabolism and angiogenesis, (Semenza, 2000). Expression in skeletal muscle of less well studied genes includes: tenascin C (TnC, mechano-regulated extracellular matrix protein associated with remodelling, (Fluck *et al.*, 2008)), bradykinin receptor type 2 (BK2, mediates most of the physiological effects of the bradykinin, which is an important indirect vasodilatory factor (Figueroa *et al.*, 2001)), high molecular weight kininogen (HMWK, the precursor to Bradykinin (Offermanns, 2008), angiotensin converting enzyme (ACE, a key regulatory enzyme which produces the body's major vasoconstrictory peptide angiotensin 2 – Ang2, (Sturrock *et al.*, 2004)), and angiotensin converting enzyme type one receptor (AT1R, main Ang2 binding receptor eliciting the classical actions of the RAS, (Baker & Kumar, 2006; Fyhrquist & Saijonmaa, 2008).

In search of master regulators of muscle phenotype: Based on screening of 25,000 possible factors encoded in the human genome a few distinct candidates for main genetic influences evolve from association of polymorphisms with fitness (Wolfarth, 2001). Chapter one section 1.1.5 provides more detail, especially Tables 6 and 7, with

linked text. One of these polymorphisms is the angiotensin converting enzyme insertion/deletion polymorphism (ACE I/Dp). It is the presence of an *Alu* insertion sequence (I-allele) and its association with elevated endurance performance and its trainability that has generated much interest over the last 20 years. See section 1.2.1 for an overview of the ACE I/Dp.

Mechanism of ACE action: The mechanism of ACE action on exercise phenotype is not known. Current investigations favour a role of its product, Ang2, as it has been shown to regulate local energy metabolism, angiogenesis, and is needed for optimal over-loaded skeletal muscle hypertrophy, via AT1R receptor (Figure 40) (Rattigan *et al.*, 1996; Gordon *et al.*, 2001; Henriksen *et al.*, 2001; Henriksen & Jacob, 2003; Dietze & Henriksen, 2008).

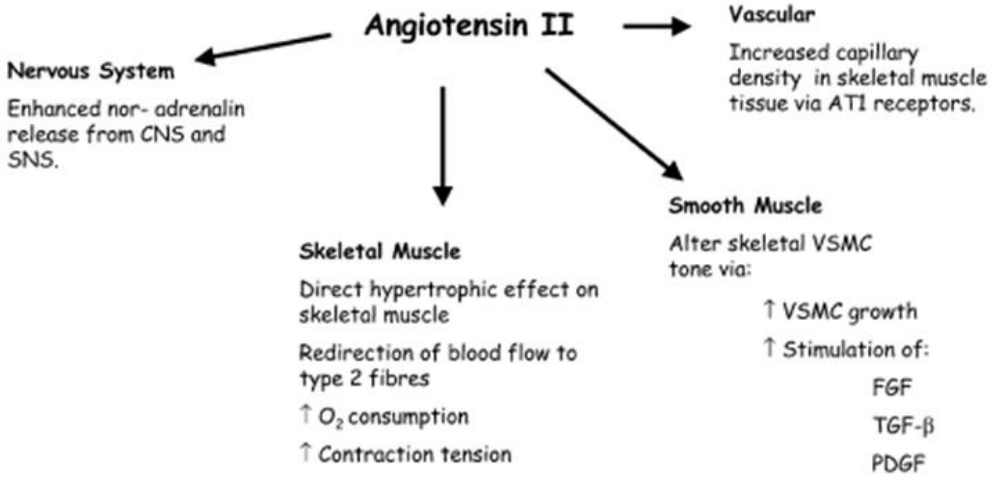

Figure 40. Effects of angiotensin 2 on muscle performance

Abbreviations: FGF, fibroblast growth factor; TGF-β, tumour growth factor-beta, PDGF, platelet derived growth factor (Jones & Woods 2003)

However, there are reports for an opposing role of Ang2 on anti-angiogenesis via AT2R (Munzenmaier & Greene, 1996). Conversely ACE also acts via breakdown of the vasodilators, the kinins (Drexler & Hornig, 1999) (Figure 41). The contribution of either of the possible ACE affected pathways and particularly to ACE modulated exercise phenotype has not been resolved: "Much of the mechanisms underlying this (ACE I/Dp importance as a single factor in the determinant of sporting performance) remain unexplored despite 12 years of research" (Puthucheary *et al.*, 2011).

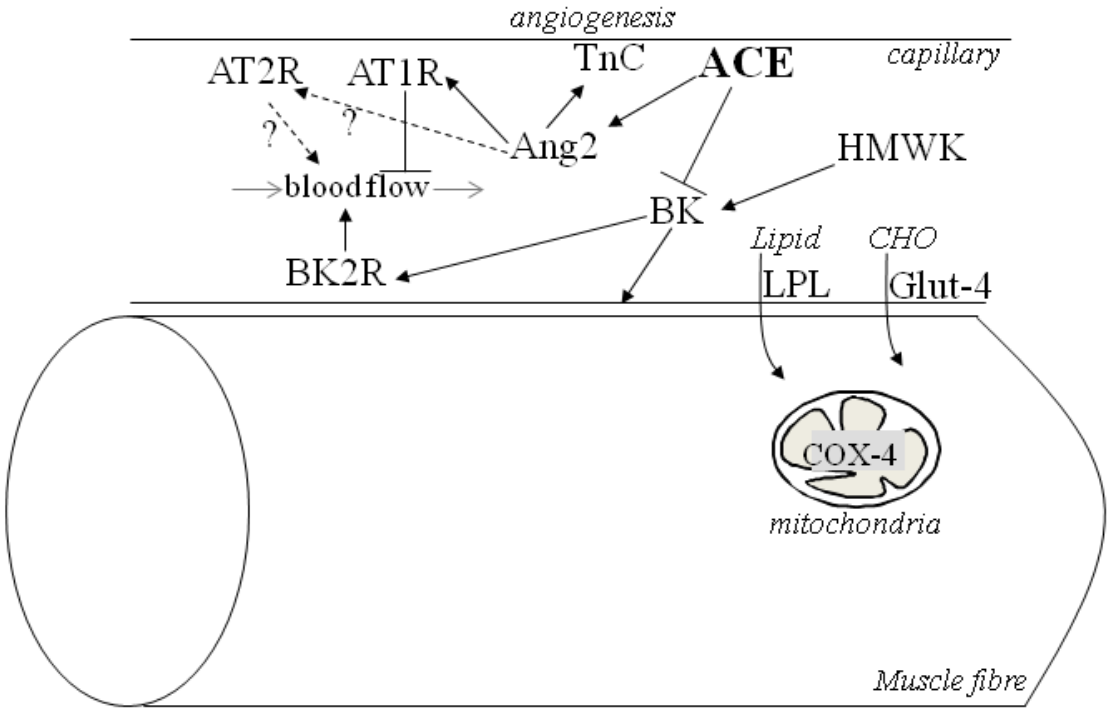

Figure 41. Hypothetical illustration of ACE signalling

Figure visualizing the hypothesized points of ACE signalling action, which were assessed at the level of transcript expression

It has been shown that altered control of gene expression in stressed muscle provides an indication of those mechanisms which are involved in signalling processes (Gracey *et al.*, 2004). If ACE controls globally the aerobic phenotype one would expect to see some evidence for this at the level of muscle structure and transcript expression (as distinct messengers encode factors that regulate metabolism and make up functional elements).

Muscle's metabolite response: As already highlighted in the introductory chapter (section 1.1.4) measuring gene expression, in isolation of other factors, can lead to misleading conclusions, as protein expression is not necessarily affected (Mata *et al.*, 2005). By using the same metabolomic approach highlighted in chapter two we were able to compare and contrast the influence, if any, of the ACE I/Dp on global polar and non-polar metabolites at rest, following an acute exercise test, and between training states – in the local muscle.

We hypothesized: that the ACE I/Dp modifies the muscle phenotype and its metabolic response to endurance exercise and that these effects would be modulated by training.

4.2. Methods

4.2.1. Experimental Design

The above hypothesis was tested by assessing the effect of the absence or presence of the ACE I-allele on muscle anatomy and the response of muscle metabolites and gene expression to a bout of endurance exercise. Towards this end we assessed to which extent elements of Ang2 signalling processes (TnC, AT1R, AT2R, ACE, LPL, BK2R, HWMK, COX-IV, GLUT-4, Figure 41) show different transcript expression in skeletal muscle, at rest and in recovery from exercise, due to the absence of ACE I-allele.

The study design was intended to expose the relation of the local muscle and system metabolic response, with a single bout of one legged endurance exercise (see 2.2.1 for rationale of using one legged testing) on a bicycle, to the ACE I/Dp and the interaction with training status and its major product Ang2. The same participants as reported in chapter two were assessed but grouped based on genotyping for the ACE I-allele. The experimenter was blinded to each participants ACE I/Dp genotype until all tests and experiments had been completed. Figure 42 illustrates the blinding protocol.

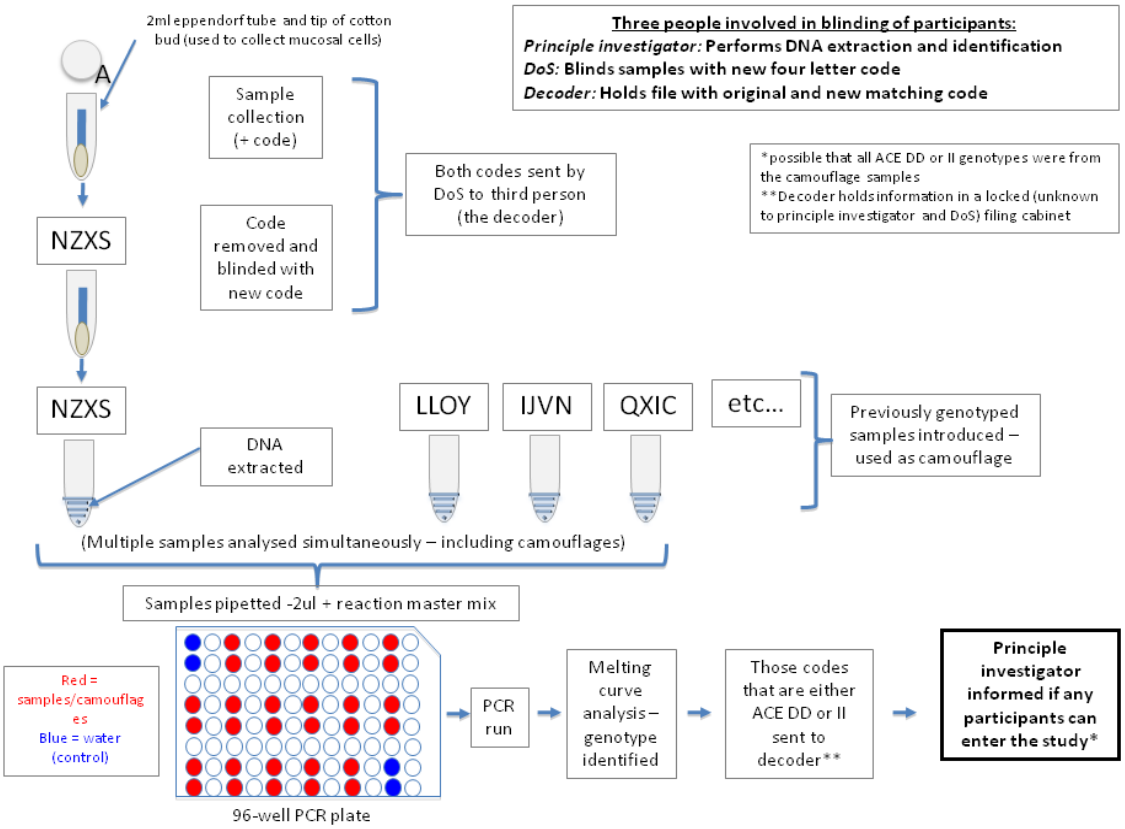

Figure 42. ACE I/Dp genotype blinding protocol

DoS: Director of StudiesProtocol and overview of how the author performed the ACE I/Dp genotyping without knowing which sample belonged to which participant. Blinding refers to the fact that the principle investigator was never informed of the ACE I/Dp genotype of each sample until after all tests/experiments had been completed. The four letter codes (e.g. NZXS) refer to the code generated by the person that anonymises (blinds) the samples

Rationale for tests

Rationale for the tests is basically the same as in chapter 3 (section 3.2.1). The rationale for choosing the single transcripts of interest is explained above (see Figure 41) and the sequences outlined below in Table 19.

Table 19. mRNA primer sequences used for real-time PCR.

Transcript	Transcript name	Genebank	Forward primer	Reverse primer
ACE	Angiotensin Converting Enzyme	NM_000789	5' TCACTACGGGGCCCAGCACA 3'	5' TGCGCCCACATGTTCCCCAG 3'
AT1R	Angiotensin 2 type 1 receptor	NM_031850	5' GTCCCGCCTTCGACGCACAA 3'	5' AACTGGCCAAGCCTGCCAGC 3'
AT2R	Angiotensin 2 type 2 receptor	NM_000686	5' CGTCCCAGCGTCTGAGAGAACG 3'	5' CACACTCCTTCAAAATTCAGGCTGC 3
BK2R	Bradykinin type 2 receptor	NM_000623	5' GGTGGGGACGGTGGGGACAT 3'	5' GGCCACACCTTCTCCGGAGC 3'
HMWK	High molecular weight kininogen	NM_001102416	5' GAACGTGACCAAGGGCATGGGC 3'	5' ACATGGCCCCCTTGGTGTTCA 3'
LPL	Lipoprotein lipase	M15856	5' TGCCCTAAGGACCCCTGAA 3'	5' AGAGTCCGTGGCTACCTGTC 3'
COX-4	Cytochrom oxidase subunit 4	X54802	5' GCCATGTTCTTCTTCATCGGTTTC 3'	5' GGCCGTACACATAGTGCTTCTG 3'
TnC	Tenascin-C	NM002160	5' ACCTCTCTGGAATTGCTCCCA 3'	5' CATCTGAAACTAGAAGGTTGTC 3'
GLUT-4	Glucose transporter type 4	M20747	5' AGCCCCCGCTACCTCTACA 3'	5' GTCAGGCGCTTCAGACTCTTTC 3'
28S rRNA	Ribosomal 28S RNA	M11167	5' ATATCCGCAGCAGGTCTCCAA 3'	5' GAGCCAATCCTTATCCCGAAG 3'

All transcripts were detected with the exception of AT2R, BK2R and HMWK.

4.2.2. Participants

Twenty healthy males (with no known medical problems) aged 19 – 38 participated in this study. Exclusion criteria included smoking, long-term ill-health, female, and not within the study age range (18 – 39). Participants all gave their written informed consent before undertaking any testing. The study and all tests were approved by the ethics committee of Manchester Metropolitan University.

Participants were deemed to be trained if they had a VO_2max >50ml/min/kg unless they deemed themselves unfit and sedentary or had indicated, by questionnaire and orally, the only activity they did was walking. This was the case of two of the untrained participants who had a VO_2max > 50ml/min/kg.

The methodology is listed below. A full description of study methodology can be found in the methods chapter (2).

4.2.3. List of all Tests/Measures (see methods chapter for description)

Same tests/measures in section 3.2.3.
In addition the following tests were carried out relevant to this study:
ACE I/Dp genotyping
ACE I/Dp related transcript analysis

4.2.4. Statistics

An unpaired test (Student's T-test) was used to compare ACE I/Dp genotype physiological and local muscle differences.

A three way ANOVA was run to expose effects of the repeated factor, post versus pre exercise, and its interaction with the "ACE -allele" and "training state" using Statistica 9.1 using post-hoc test of Fisher (Statsoft, Tulsa, USA). ACE I/Dp genotype effects at rest and its interaction with training were assessed with a two-way ANOVA.

4.3. Results

ACE I/Dp dependent exercise phenotype and interaction with training state: 20 prticipants were investigated for ACE genotype dependent physiological variables of exercise capacity. The Hardy-Weinburg Equilibrium (HWE), which tells us about the relataive frequencies of alleles (and variability/stability from generation to generation), has been shown to be consistently met in studies investigating the ACE I/Dp (Montgomery *et al.,* 1998; Hagberg, *et al.,* 2002). However, low number of samples (participants) is often quoted for not being able to calculate the HWE – our study sample number was low (n=20), therefore it was not possible to calculate whether the HWE was met. However, when all participants (n = 60) who were genotyped were taken into consideration (II 13.3 %: ID 66.7%: DD 20.0%) the HWE was satisfied. Table 20 lists the ACE I-allele dependent participant characteristics.

Table 20. Participant characteristics grouped by ACE I/Dp genotype.

	ACE IDp genotype				
	DD (n = 11)		ID + II (n = 9)		P
Age (years)	25.2	(19:36)	27.3	(21:38)	0.46
Body mass (Kg)	75.5	(60:93)	79.8	(65:99)	0.38
Height (M)	1.81	(1.67:194)	1.81	(1.77:1.91)	0.97
BMI (Kg/M^2)	23.0	(18.5:27.6)	24.3	(20.3:31)	0.37
Body fat (%)	17.4	(8.8:29.4)	17.8	(6.1:32)	0.90
VO_2max (ml/min/Kg): 2-leg	54.5	(43.2:70.1)	55.3	(46.9:66)	0.83
VO_2peak (ml/min/Kg): 1-leg	44.8	(34.2:52.2)	46.6	(41.2:56.0)	0.49
VO_2max (ml/min): 2-leg	4101.7	(3217:5319)	4385.1	(3115:5288)	0.44
VO_2peak (ml/min): 1-leg	3377.1	(2397:4719)	3700.7	(2677:4616)	0.26
Pmax (W): 2-leg	301.4	(230:410)	348.9	(250:450)	0.11
Pmax (W): 1-leg	193.6	(140:260)	217.2	(145:275)	0.22
Pmax (W/Kg): 2-leg	3.98	(3.31:4.67)	4.40	(3.43:5.42)	0.16
Pmax (W/Kg): 1-leg	2.56	(2.00:3.40)	2.73	(2.22:3.41)	0.39
RER: 2-leg (start of exercise)[a]	0.81	(0.71:0.87)	0.79	(0.72:0.86)	0.32
RER: 2-leg (end of exercise)[a]	1.09	(0.96:1.17)	1.00	(0.99:1.18)	0.42
RER: 1-leg (start of exercise)[a]	0.78	(0.67:0.90)	0.75	(0.65:0.83)	0.39
RER: 1-leg (end of exercise)[a]	1.06	(0.92:1.22)	0.98	(0.99:1.15)	0.67

There were no significant differences at the physiological level between the genotypes. Assessed by unpaired Student's T-Test. [a]DD n=8, [a]II/ID = 8

There were no significant interactions between 'ACE I/Dp genotype' and 'training status' (including age) on any of the assessed physiological variables of exercise performance (2-way ANOVA for factors 'presence of the ACE I-allele' x 'training status').

ACE dependent muscle phenotype: There was a significant effect of the presence of the ACE I-allele on muscle capillarity. Participants with the ACE I-allele showed

Table 21. Participant muscle characteristics grouped by ACE I/Dp genotype.

	ACE I/Dp genotype				
	DD (n = 11)		**ID + II (n = 9)**		**P**
Quadriceps volume (cm^3)	2075.1	(1419:2919)	2290.3	(1672:2949)	0.29
Capillary density (mm^{-2})	277.3	(207:345)	311.4	(282:352)	**0.04**
Type I fibre (%)[a]	41.81	(34.80:52.60)	41.5	(38.0:63.0)	0.96

Capillary density (mm^{-2}) significantly different greater in genotype with the I-allele (P=0.04, assessed by unpaired Student's T-test). [a]DD n = 10

greater capillary density in the *vastus lateralis* muscle than those without the I-allele (p=0.04). The training state did not demonstrate a significant interaction with this genotype difference (p=0.25).

ACE I/Dp dependent muscle metabolite response: We assessed whether the ACE-dependent difference in muscle capillarity would be reflected by an altered response of metabolites in serum and at the level of the recruited *vastus lateralis* muscle. From the panel of selected serum metabolites of glucose and lipid metabolism, which were measured immediately after the exercise, LDL concentration demonstrated a significant interaction effect of the 'exercise response' x 'ACE I-allele' x 'training state' (p=0.03).

Post hoc testing resolved that LDL was significantly reduced in trained participants without the I-allele (p=0.04).

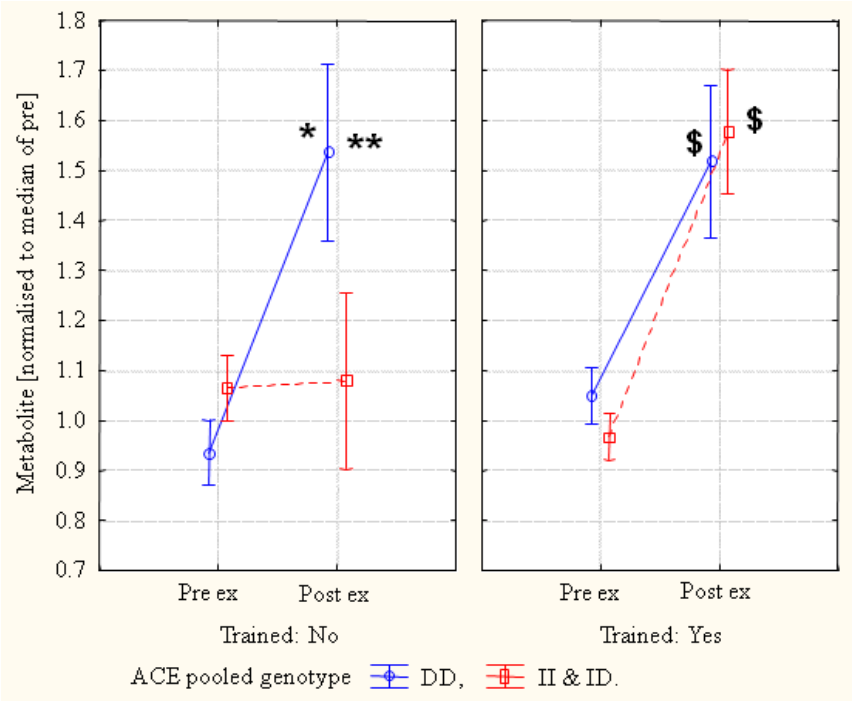

Figure 43. Interaction effect of ACE I/Dp genotype and training status on the exercise response of non-polar metabolites

*P=0.001, Repeated ANOVA, vs. pre exercise without the I-allele. **P=0.001, Repeated ANOVA, vs. post-exercise with the I-allele. $P=0.001, Repeated ANOVA, vs. pre exercise with or without the I-allele. Vertical bars denote 0.95 confidence intervals.

Normalised data to pre-exercise (T & UT) = 1.0

We assessed the local changes of polar and non-polar metabolites in *vastus lateralis* muscle 30 minutes after single leg exercise with mass spectrometry. There was an interaction effect, between 'ACE I-allele' x 'post exercise response' (P=0.009) and between 'ACE I-allele' x 'post exercise response' x 'training state' (P=0.0002), for non-polar metabolites (Figure 43). Post hoc tests showed that the selective increase in non-polar metabolites in ACE-DD genotypes in untrained participants is lost in trained participants as both genotypes demonstrate an increase.

Similar observations were made for the exercise induced changes in polar metabolites assessed with LCMS in positive (LCMS_ESI+, Figure 45) and negative (LCMS_ESI-, Figure 44) mode, although using LCMS_ESI+ this interaction was not significant.

Interestingly, the genotype effects on the exercise response of muscle metabolites in the untrained state were all lost in trained participants (Figure 45).

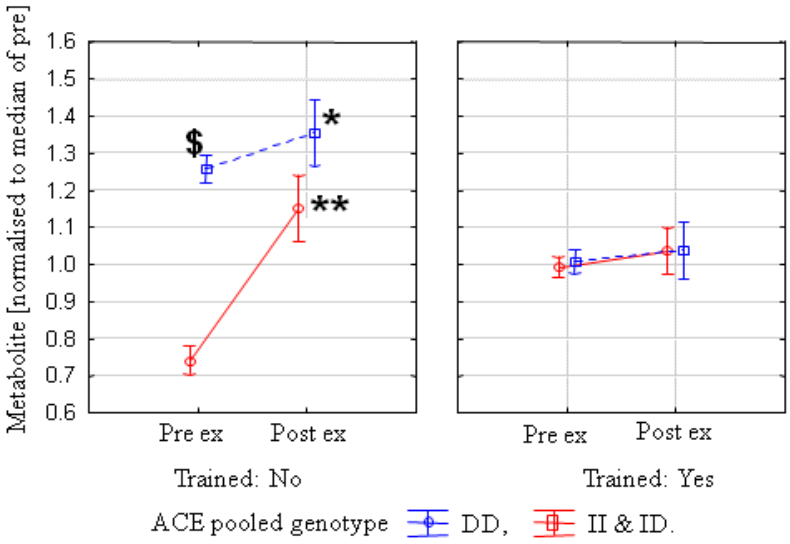

Figure 44. Interaction effect of ACE I/Dp genotype and training status on the exercise response of polar metabolites (LCMS_ESI-).

*P<0.05, Repeated ANOVA, vs. with I-allele post exercise. **P<0.001, Repeated ANOVA, vs. pre-exercise with I-allele. $P<0.001, Repeated ANOVA, vs. with I-allele pre-exercise. Vertical bars denote 0.95 confidence intervals.

Normalised data to pre-exercise (T & UT) = 1.0

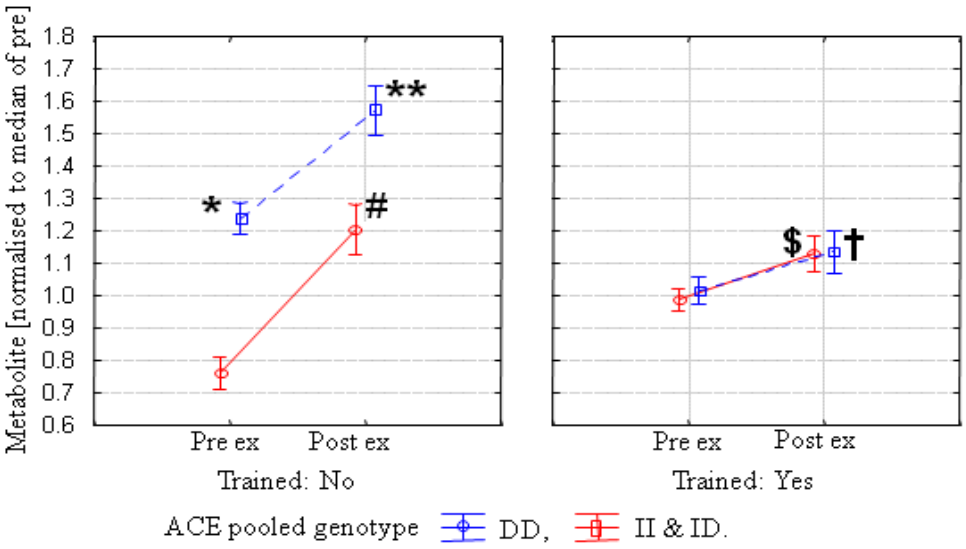

Figure 45. Interaction effect of ACE I/Dp genotype and training status on the exercise response of polar metabolites (LCMS_ESI+)

*P<0.001, Repeated ANOVA, vs. pre-exercise with the I-allele. **P<0.001, Repeated ANOVA, vs. pre-exercise without the I-allele. #P<0.001, Repeated ANOVA, vs. pre-exercise with the I-alelle. $P<0.001, Repeated ANOVA, vs. pre-exercise with the I-allele. †P<0.05, Repeated ANOVA, vs. exercise without the I-allele. Vertical bars denote 0.95 confidence intervals.

Normalised data to pre-exercise (T & UT) = 1.0

ACE I/Dp dependent muscle gene response: We analyzed the effect of the ACE I/Dp genotype on combined transcript expression in *vastus lateralis* muscle using reverse-transcriptase polymerase chain reactions (rt-PCR). A number of gene transcripts for factors of Ang2 signalling (AT1, AT2, BKR2, HMWK, Tn-C) and (ACE dependent factors) involved in substrate import from the capillaries (Glut-4, LPL) and oxidative metabolism (COX-4) were selected. All were detected except AT2, BKR2 and HMWK – it's possible they were not present or below the dection limit.

There was a general interaction between "ACE I/Dp genotype" x "training state" on expression of the selected transcripts at rest (P=0.02, Figure 46).

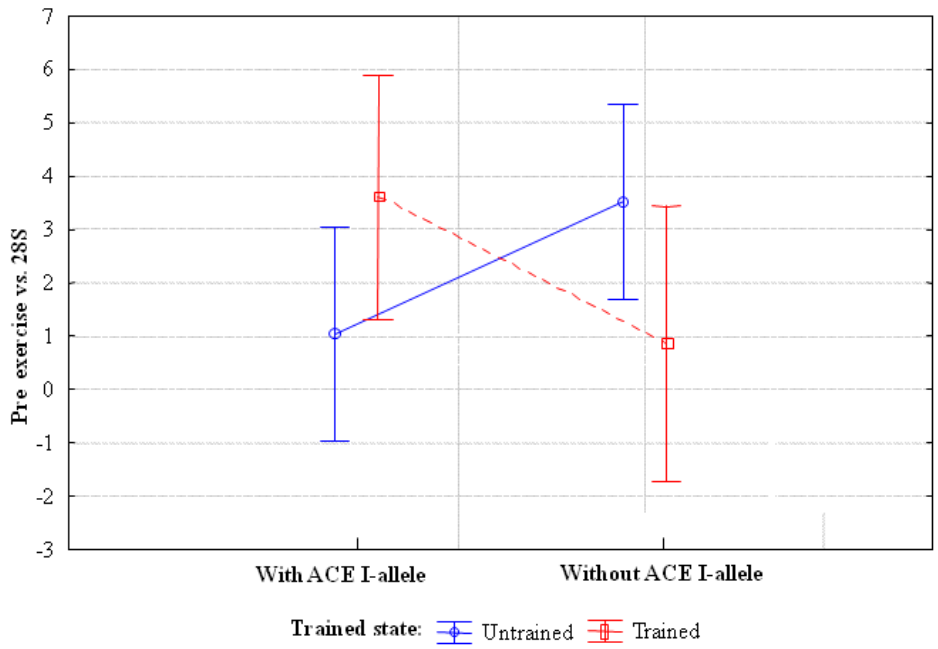

Figure 46. Interaction of ACE I/Dp genotype and training status of combined muscle transcripts

P=0.02, Two-way ANOVA, ACE I/Dp genotype x training state, combined muscle transcripts: ACE, AT1R, TnC, GLUT-4, LPL, COX-4. Vertical bars denote 0.95 confidence intervals.

In untrained participants there was a significant interaction effect between 'ACE genotype' and the response to exercise when all transcripts were assessed combined (P=0.05). This interaction effect did not reach the level of statistical significance in trained participants (P=0.184).

A detailed inspection of the ACE I/Dp genotype identified single transcripts that were modified (Figures 47, 48). Significant effects for single gene transcripts involved higher ACE mRNA level in *vastus lateralis* muscle of untrained participants without the ACE I-allele, and a significant reduction eight hours post exercise (Figure

47). Also, LPL mRNA was reduced in untrained participants without the ACE I-allele eight hours post exercise (Figure 47). Both these differences were lost in the trained state (Figure 48).

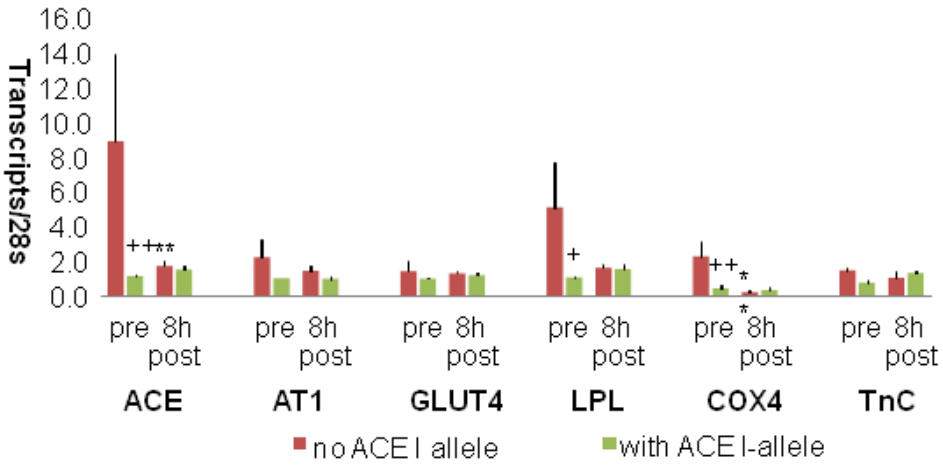

Figure 47. Pre- and post-exercise effects for each muscle transcript between ACE I/Dp genotypes in untrained participants.

Transcripts [per 28s rRNA], untrained cohort.

$^{+}$: 0.05< p <0.10vs. pre other genotype, $^{++}$: p< 0.05 vs. pre other genotype, *: 0.05< p <0.10vs. pre same genotype, **: p< 0.05 vs. pre same genotype. MANOVA for the repeated factor 'time respective to exercise' (i.e. pre vs. 8 h post) with a post hoc test of Fisher. Mean and standard error of muscle transcript levels pre and post exercise.

TnC mRNA also showed elevated expression in genotypes with the I-allele in trained participants only (p=0.02, Figure 48), but this difference was lost eight hours post exercise. The changes in transcript expression eight hours into recovery demonstrated a general interaction effect with the 'presence of the ACE I-allele' and training state (p=0.04). When the ACE I/Dp genotype dependent effect was assessed for each transcript only for COX-4 was this interaction preserved (p=0.07).

There was a generally elevated level of transcript levels in untrained ACE I/Dp genotypes without the I-allele pre-exercise (p=0.03). This was lost post-exercise.

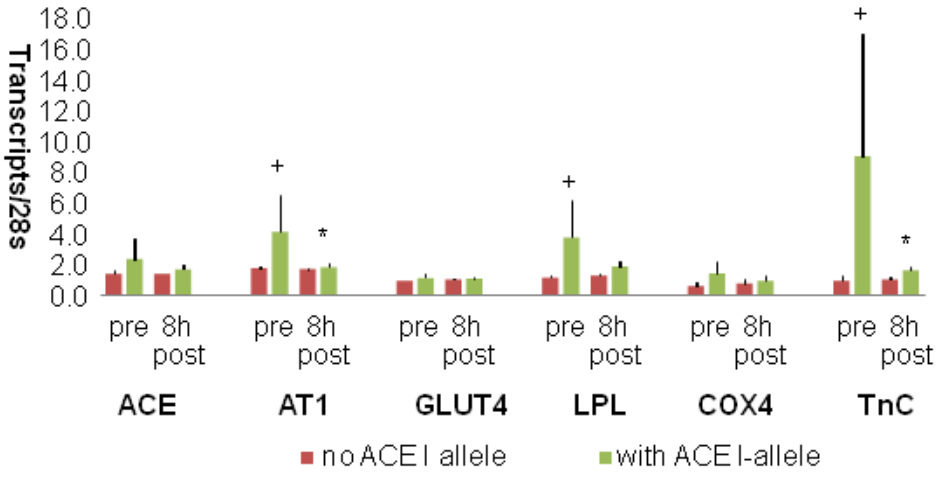

Figure 48. Pre- and post-exercise effects for each muscle transcript between ACE I/Dp genotypes in trained participants

Transcripts [per 28s rRNA], trained cohort.

$^{+}$: 0.05< p <0.10vs. pre other genotype, *: 0.05< p <0.10vs. pre same genotype. MANOVA for the repeated factor 'time respective to exercise' (i.e. pre vs. 8 h post) with a post hoc test of Fisher. Mean and standard error of muscle transcript levels pre and post exercise.

4.4 Discussion

The insertion polymorphism in intron 16 of the ACE gene has firmly been related to endurance performance and the training effect of exercise (Montgomery *et al.*, 1998; Williams *et al.*, 2000). The contribution of a muscle mediated mechanism to this phenomenon is not understood (Puthucheary *et al.*, 2011). We show that this ACE I/Dp influences both the molecular and metabolite response to an acute bout of exercise, and that local delivery vessels are altered. Plus, we identified that the ACE I/Dp modulates muscle metabolites, non-polar and polar differently, as a consequence of the environmental stimulus of regular endurance exercise (trained state). There was a blunting of muscle polar metabolites in the trained population (Figures 44, 45). However, the blunting of non-polar muscle metabolites was over-ridden in the trained population with the I-allele, mirroring the trained population without the I-allele (Figure 43). Thus, the environmental stimulus of regular exercise (trained population)

over-rides the ACE I/Dp effects on muscle metabolite metabolism during recovery from an acute bout of endurance exercise.

As our study was limited by a cross-titudinal design there is uncertainty whether the interaction of training and ACE I/Dp on the metabolome and transcript levels is explained by a direct effect of the repeated exercise. Thus, the general ACE I/Dp dependent regulation of transcript expression of factors involved in Ang2-dependent signal processes, and key parameters involved in import of carbohydrate and lipid factors at rest and following endurance exercise is of interest (Figures 47 and 48). There is support for a role of the environmental stimulus of exercise over-riding the genetic influence of the *Alu* insertion sequence in the ACE gene, due to the inversion of the ACE I-allele dependent exercise response. Due to absent interaction effects of exercise and ACE I/Dp on single transcripts it is not possible to draw definite conclusions on ACE I/Dp control of the Ang2/RAS system. Bearing this in mind it is of interest to note the ACE I/Dp genotype effect on the two transcripts, ACE and TnC. The higher ACE mRNA levels in ACE DD genotypes hints for the existence of a local ACE I/Dp modulated RAS system in skeletal muscle which expression is regulated by training. Equally it also suggests a role for TnC as downstream effector of ACE as indicated previously in cell culture (Hahn *et al.*, 1995).

By contrast, our data does not support that altered expressional regulation of receptors downstream of ACE, Ang2 or BK signalling (i.e. BK2R, AT1R and AT2R) is involved in muscle's adaptation to endurance training. In fact, the undetected amplification of AT2R and main kinin receptor (BK2R) transcripts using RT-PCR with indicate a possible minor contribution of this system to ACE effects in the studied muscle.

Our metabolomic scan identified a general effect of the ACE I/Dp on exercise-induced changes in metabolite levels in both polar and non-polar fractions of the exercised vastus lateralis muscle (Figures 43, 44, and 45). This implies that the ACE I/Dp influences variability in muscle’s metabolic response to exercise. A number of factors which warrant further exploration could account for this. One, possibly major contributor, is a net effect on blood vessels (perfusing muscle) capacity to vasodilate and therefore downstream effect on angiogenesis. This contention of a possibly altered perfusion is in line with the significant difference in capillary density in vastus

lateralis between ACE I/Dp genotypes, and the supply dependent muscle metabolome after a workout.

Interestingly, the ACE I/Dp dependent change in the polar fraction were completely blunted in trained participants (Figures 44, 45). These findings imply an important fraction of the ACE I/Dp genotype effect on metabolite flux is modified dependent on whether participants regularly perform physical activity. This finding suggests that regular exercise overrides to some extent the genetic inhibition of biological processes downstream of ACE which is evident at a muscle level at rest.

4.5. Conclusion

From this small study, it appears that an *Alu* insertion sequence is possibly influencing different system level responses to an acute exercise bout. An aerobic phenotype would favour greater vascular space (capillary density) at the local working muscle to deliver and remove oxygen/carbon dioxide and energy substrates, which was evidenced in participants with the *Alu* insertion sequence. Participants without the insertion sequence (ACE DD) demonstrated: reduced capillary density, a greater reliance on local lipid metabolism (increased non-polar metabolites pre and post exercise) in the untrained state, and had reduced levels of LDL following an acute exercise bout in the trained state.

These results tentatively suggest that this *Alu* insertion sequence, in an important regulator of a key gene (ACE), which controls a major vasoconstrictory peptide (Ang2), favours the development of an aerobic muscle phenotype.

Chapter 5

Stability of the ACE modulated muscle phenotype between populations with a specific ACE variant

5.1. Introduction

Endothelial cells play a vital role in substrate delivery to the working muscle, interacting and responding to local changes (Clifford & Hellsten, 2004). A key function of the endothelial cells is their role in altering blood flow by dilation/constriction. The ACE I/Dp may be involved in regulating substrate supply to working muscle, therefore it will influence the conditioning of metabolic fitness by endurance exercise. As we, and others, have shown in the previous chapter this involves capillary supply lines in muscle as one of the most plastic structures (Hudlicka *et al.*, 1992). Whilst the literature is not conclusive there is evidence that reduced ACE activity is associated with a greater vascular space (Dietze & Henriksen, 2008), increased glucose usage (Dietze & Henriksen, 2008; Jayasooriya *et al.*, 2008), and a more reactive vasodilatory capacity (Brown *et al.*, 1998b). This relationship is replicated with ACEi's and in the presence of the insertion sequence – ACE II/ID (Buikema *et al.*, 1996; Brown & Vaughan, 1998a; Brown *et al.*, 1998b; Butler *et al.*, 1999; Arcaro *et al.*, 2001; Henriksen & Jacob, 2003). Whilst ACE is found circulating free in plasma, the majority is (Danser *et al.*, 2007) is anchored to endothelial cells (Figure 26 B). An often overlooked effect of increased ACE activity (when the *Alu* insertion sequence is missing) is not only an amplified production and/or conversion of Ang1 to Ang2, but also an increased degradation of BK (a potent dilatory factor) (Brown *et al.*, 1998b; Murphey *et al.*, 2000). Much of the literature on ACE I/Dp and human performance looks into the effects of the *Alu* insertion sequence and trainability or increased/decreased frequency in differently trained populations versus untrained controls (Montgomery *et al.*, 1998; Myerson *et al.*, 1999; Rankinen *et al.*, 2000b; Hagberg *et al.*, 2002; Scanavini *et al.*, 2002; Hruskovicova *et al.*, 2006; Pescatello *et al.*, 2006; Thompson *et al.*, 2006). Due to the plethora of factors influencing human performance it is not surprising to find much controversy, on the ACE I/Dp, in the literature. The influence (if it really exists) of the ACE I/Dp will likely be small but potentially significant due to the key end products it effects: Ang2 and BK. Studies have demonstrated that Ang2 increases with exercise (Staessen *et al.*, 1987; Miura *et al.*, 1994; Woods *et al.*, 2004). However, sustained exercise (over 3 minutes) over-rides constriction (Murrant & Sarelius, 2000). Therefore can training over-ride any influence of the ACE I/Dp, and if so at what system level? We found in chapter three

that training state 'inverted' ACE I/Dp dependence, but whether this was due to repeated endurance type exercise per se or the natural habitat of the participants is not known.

A potentially important factor contributing to the ACE I/Dp controversies is the consistency of its influence between different ethnic groups (Scott *et al.*, 2005; Payne *et al.*, 2007; Woods, 2009). Therefore the role played by the genetic background and habitual factors probably interacts with other genes x environment on the ACE-related phenotype. These interactions are not related to ethnic differences in the ACE I/Dp allele frequencies, per se (see introduction – Figure 31).

We hypothesised: that the exercise induced pathway of ACE related muscle signalling is preserved, in different populations, as this environmental stimulus reflects a dominant perturbation of the Ang2 signalling pathway.

However, steady state differences in physiological variables relating to the trait of metabolic fitness between genotypes of the ACE I-allele may not necessarily be preserved between populations (see section 1.2.6 for a brief explanation of differences in the ACE I/Dp and ethnicities). This was tested by comparing a Swiss and British population – even though both populations are caucasion, their culture, habitat (altitude) and ancestry is different – (over ten's of thousands of years and beyond).

5.2 Methods

5.2.1. Experimental Design

Using an integrative approach two cohorts of healthy, male Caucasians were investigated: Swiss and British cohorts. Both cohorts were analysed for the ACE I/Dp genotype, and their gene expression response and organelle content, in relation to energy substrate usage and pathways, in recruited *vastus lateralis* muscle at rest. This muscle tissue was chosen due to its important recruitment during the exercise tests on the bicycle (Krustrup *et al.*, 2004b).

In addition a subset of the Swiss cohort was also assessed following an endurance training regime, and the British cohort was split into trained and untrained subsets. Whole body aerobic capacity was also assessed.

Rationale for tests

Rationales for tests (for the British cohort) is the same as chapter 3 (section 3.2.1). As the Swiss data came from study data already analysed (see 5.2.2.) the only extra test required was the ACE I/Dp genotyping. This analysis was carried out in collaboration with Prof. Hans Hoppeler from the University of Berne. The author's role in this specific part of the study was limited to the ACE I/Dp genotyping of each sample, which was performed using preserved skeletal muscle material (see chapter 2 – methods, and 5.2.3. below).

5.2.2. Participants

Age, height, body mass, and percent body fat were recorded for all participants in both the Swiss and British cohorts.

The Swiss cohort comprised two not systematically trained, Swiss populations (males) from the Canton of Berne (Switzerland), from two studies which underwent an endurance program composed of 6 weeks of bicycle exercise or 6 months of jogging as described were included in the analysis (Suter *et al.*, 1995; Schmutz *et al.*, 2006; Schmutz *et al.*, 2010). The investigations were conducted with permission of the Ethics Committee of Bern, Switzerland, in compliance with the Helsinki Convention for Research on human participants.

The British cohort comprised 9 untrained and 11 trained males from the Manchester area of the UK (see section 3.2.2). The study and all tests were approved by the ethics committee of Manchester Metropolitan University.

Participants were deemed to be trained if they had a VO_2max >50ml/min/kg unless they deemed themselves unfit and sedentary or had indicated, by questionnaire and orally, the only activity they did was walking. This was the case of two of the untrained participants who had a VO_2max > 50ml/min/kg.

The methodology is listed below. A full description of study methodology can be found in the methods chapter (2).

5.2.3. List of all Tests/Measures (see methods chapter for description)

British Population *(Author's own research/data)*

All tests/measures have already been covered in section 3.2.3 and 4.2.3.

Swiss Population *(Berne Uinversity research/data)*

Additional tests/measures done for the Swiss population, which is already published (Suter *et al.*, 1995; Schmutz *et al.*, 2006)

Genotyping: For both cohorts, DNA isolation was performed from pooled cryosections of approximately 10 mg tissue of *m. vastus lateralis* following a commercially available protocol (Qiagen DNeasy Blood and Tissue Handbook, 07/2006, cat. no. 69504).

ACE genotyping was carried out with Polymerase chain reaction (PCR) as described by Evans et al., (1994) (Evans *et al.*, 1994). The primers cor responded to those established previously for the identification of the ACE-I/D genotype (for details see Genebank number X62855):

Detection of the 66 bp amplicon specific for the I-allele in intron 16 of the ACE gene was achieved by a combination of ACE2 (5'-tgggattacaggcgtgatacag-3') and ACE3 (5'-atttcagagctggaataaaatt-3') primers. ACE1 (5'-catcctttctcccatttctc-3') and ACE3 (5'-atttcagagctggaataaaatt-3') primers were applied to detect the 83 bp amplicon specific to the absence of the insertion sequence (i.e. the D-allele).

Whole body aerobic capacity: Maximal oxygen uptake, peak power output and whole body respiration (from respiration exchange ratio) was determined with ergospirometry in an incremental endurance test on a stationary bicycle ergometer (Ergoline 800S, Jaeger, Ergoline 800S, Ergoline GmbH, Bitz) using a Cortex system (k4b2) as described (Suter *et al.*, 1995; Schmutz *et al.*, 2006).

Endurance exercise: Participants from cohort one carried out a single bout of endurance exercise. Muscle biopsies were collected from *vastus lateralis* muscle for the purpose of profiling the transcript response into recovery from exercise as reported (Schmutz *et al.*, 2006). The resting biopsy was collected two weeks before exercise. Thereafter participants carried out a single bout of two-legged endurance exercise at

65% peak power output after an overnight fast with collection of biopsies 1, 3, 8 and 24 hours in alternating order from new incision sites in the left or right leg as reported (Schmutz *et al.*, 2010).

Participants from cohort two completed a 6-month endurance exercise training program comprising home-based jogging of 120 min/wk at 75% VO_2max for which adjustments in muscle structure and whole body aerobic capacity had been described (Suter *et al.*, 1995). The biopsies of cohort two were collected pre and post endurance training with Bergstroem needles.

Muscle structure: Quantitative alterations in muscle ultrastructure were evaluated with established morphometrical technique from glutaraldehyde fixed muscle biopsies (Schmutz *et al.*, 2006). In brief, this comprised the assessment of volume densities of myofibrils, total mitochondria, subsarcolemmal mitochondria, intramyocellular lipids, residual organelles and capillaries. Additionally muscle fibre cross section, capillary-to-fibre ratio, and fibre types were estimated (Suter *et al.*, 1995).

Transcript profiling: Level alteration of gene transcripts for major function of skeletal muscle pre and 8 hours post exercise in the untrained and trained state in the subjects were evaluated in total RNA with validated low-density Atlas® cDNA arrays (BD Biosciences, Allschwil, Switzerland; (Schmutz *et al.*, 2006). This time point was chosen as it refers to the peak response of transcript expression in untrained subjects (Schmutz *et al.*, 2010). The array platform (GPL 1935 at Gene Omnibus, http://www.ncbi.nlm.nih.gov/geo) covered 231 transcripts from the major gene ontologies (GO) underlying muscle energy and protein metabolism, fibre structure and angiogenesis. Microarray data series were deposited under accession codes GSE 13623 and GSE 2479, respectively, at GEO.

5.2.4. Statistics

Physiological and structural variables were assessed with Student's t-test. A repeated ANOVA was applied to compare 'pre vs. post' effects of exercise or training between genotypes 'with ACE I-allele' (ACE-ID/ACE-II) and 'without ACE I-allele' (ACE-DD) for the measured variables (serum metabolites, ultrastructural variables) using Statistica 9 (Statsoft).

Gene expression: Microarray data were background corrected as described (Schmutz *et al.*, 2010). Subsequently, the background-corrected signals were related to the total count of transcript signal per array to reveal normalized values. Significant adjustments in muscle transcript expression following a single bout of endurance exercise and training, between ACE genotypes with or without the I-allele were evaluated from normalized values using a Significance Analysis of Microarrays test (SAM) running as applet in Excel software (Tusher *et al.*, 2001). Default settings for two class paired data with post hoc T-tests were used during the calculation. The largest delta value, with the smallest significant false discovery rate (FDR, denoted as q-values), was set to calculate all differently expressed genes.

5.3. Results

ACE I/Dp difference in oxygen dependent pathways: British versus Swiss population: We assessed whether ACE I/Dp differences of oxygen delivery and utilisation (maximal oxygen usage: VO_2max, maximum power output: Pmax, capillary density: CD, and oxidative fibre type: Type I), seen in the British population (previous chapter, section 3.3), were preserved in a population of untrained Swiss males.

Table 22. Physiological characteristics of both cohorts: Swiss and British

Swiss Cohort	ACE I/Dp genotype				
	DD (n = 10)		ID + II (n = 10)		*P*
Age (years)	30.5	(±2.7)	32.8	(±2.4)	0.53
Height (M)	1.76	(±2.1)	1.76	(±2.6)	0.91
Body Mass (Kg)	71.6	(±2.5)	79.6	(±5.1)	0.15
BMI (Kg/M^2)	23.0	(±0.5)	25.4	(±1.5)	0.11

British Cohort	ACE I/Dp genotype				
	DD (n = 11)		ID + II (n = 9)		*P*
Age (years)	25.2	(±1.7)	27.3	(±2.3)	0.46
Height (M)	1.81	(±0.02)	1.81	(±0.02)	0.97
Body Mass (Kg)	75.5	(±2.7)	79.8	(±4.0)	0.38
BMI (Kg/M^2)	23.0	(±0.8)	24.3	(±1.2)	0.37

Numbers represent mean (SE±). Unpaired Students T-Test.

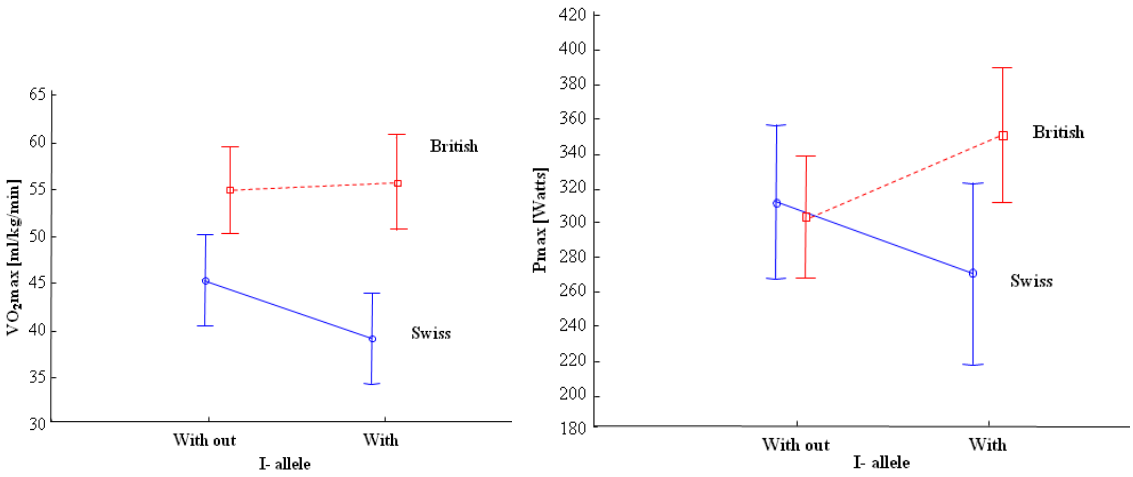

Figure 49. Maximal oxygen uptake and power output, without and with the I-allele, in British and Swiss populations

Vertical bars denote 0.95 confidence intervals.

Effect of ACE I/Dp genotype x population (VO_2max and Pmax).

VO_2max x ACE I/Dp genotype x population: p=0.15 (Repeated ANOVA). Pmax x ACE I/Dp genotype x population: p=0.05 (Repeated ANOVA).

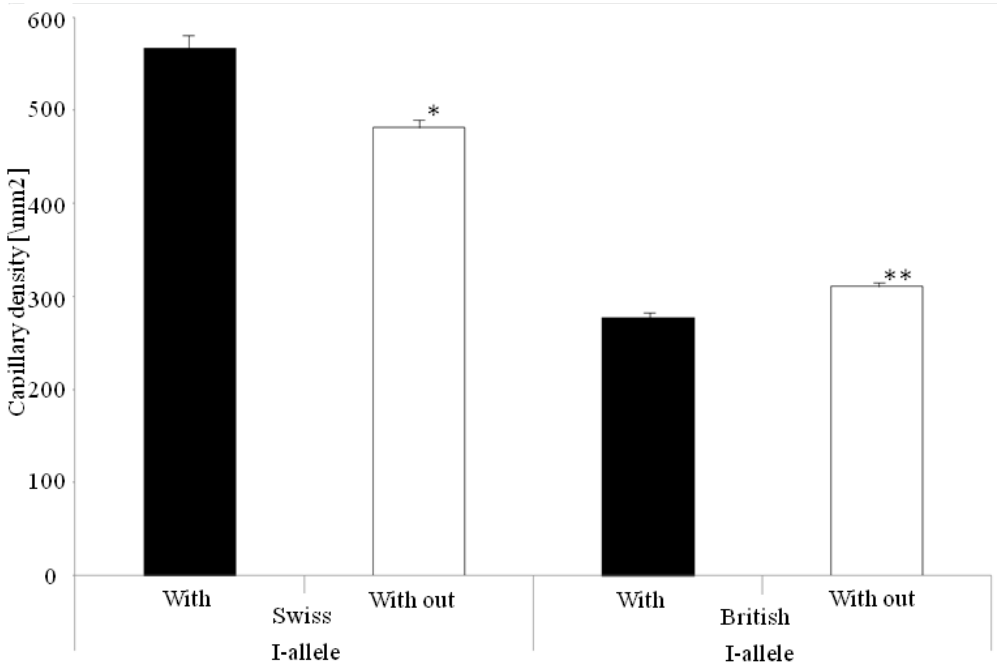

Figure 50. Capillary density, without and with the I-allele, in British and Swiss populations

*p=0.10. **p=0.04. Swiss n=10 with, and n = 10 without I-allele; British n = 11 with, and n = 9 without I-allele. Unpaired Students T-Test. (Error bars ± SEM). Swiss population biopsies taken with chonchotome – mid point of *vastus lateralis*; British population biopsies taken with fine-needle mid point of *vastus lateralis*.

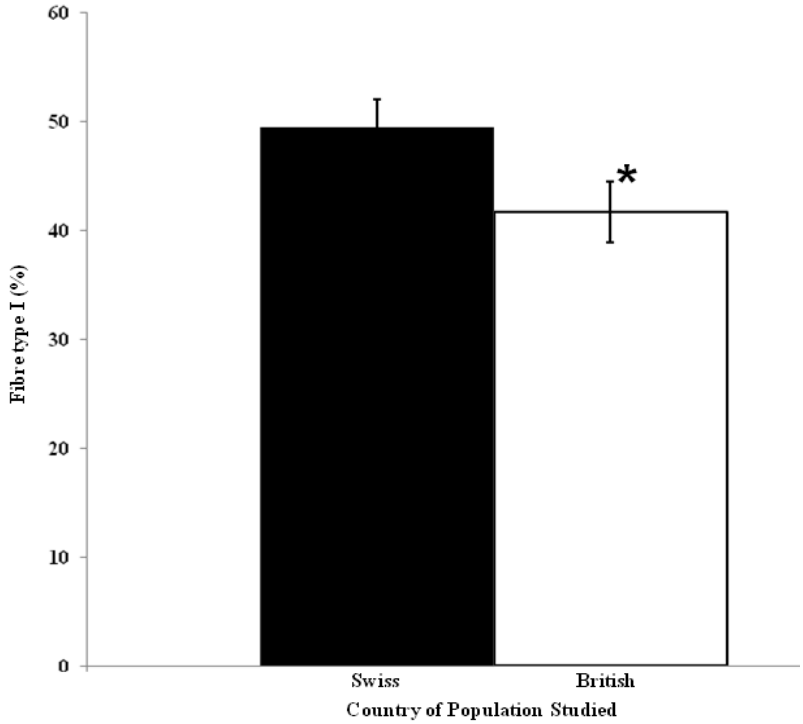

Figure 51. Type I muscle fibre differences, in British and Swiss populations (not separated by ACE I/Dp)

*p=0.06. Unpaired Students T-Test. Error bars ± SEM.

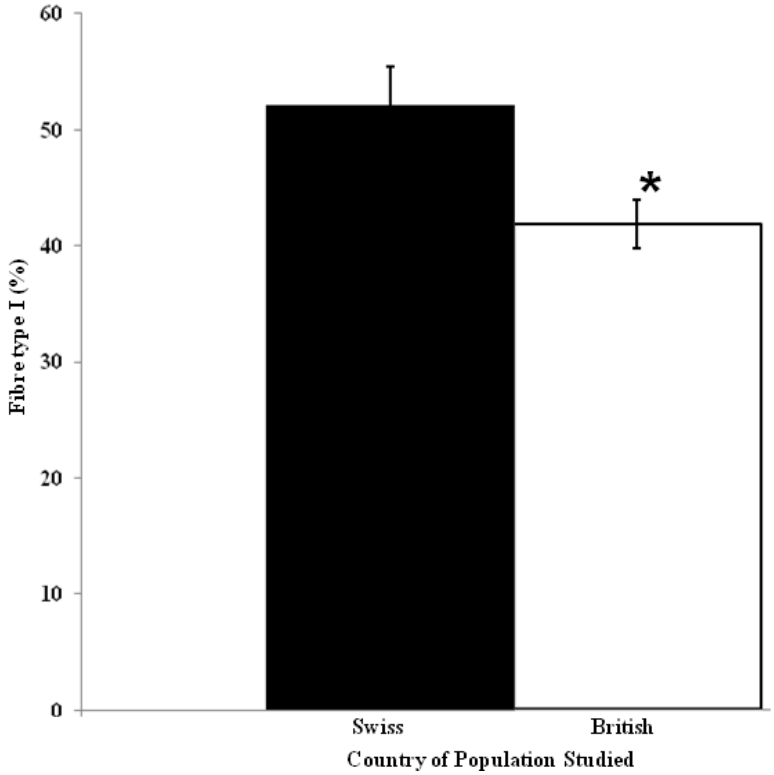

Figure 52. Type I muscle fibre differences, in British and Swiss populations without the I-allele.

*p=0.02. Unpaired Students T-Test. Error bars ± SEM.

We explored whether exercise, which controls the pathway of oxygen utilisation and the product of the ACE gene (Ang2), is a factor in explaining these differences. Therefore we sought to uncover whether the ACE I/Dp genotype dependent muscle response of transcript expression, seen in the British cohort during recovery from a bout of endurance exercise (see chapter three), is preserved in Swiss participants of this study. The previously investigated factors of Ang2 signalling (ATR1, ATR2, BKR1, HWMK, TnC) and ACE dependent factors involved in substrate delivery from capillary (GLUT-4 and LPL) and oxidative metabolism (COX-4) were analysed in a previously published set of microarray data (Schmutz *et al.*, 2006; Schmutz *et al.*, 2010).

There was a trend, in the British cohort, for a larger increase in expression of (all) transcripts in the *vastus lateralis* muscle eight hours post exercise, in participants with the I-allele (p=0.07, ANOVA) (Figure 53). This trend for a larger increase of (all) transcripts was reproduced in the Swiss (p=0.06, ANOVA) (Figure 53).

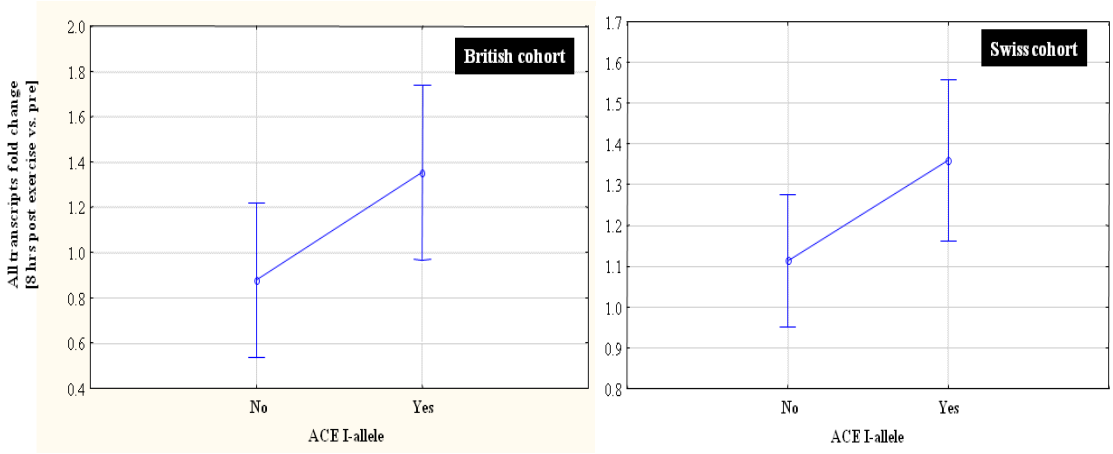

Figure 53. Fold changes in combined gene transcripts (pre- versus post-exercise) in untrained British and Swiss cohorts, split by ACE I/Dp genotype (please note scales not identical)

British cohort: p=0.07, Swiss cohort: p=0.06

Effect of ACE I/Dp genotype (ACE ID/ID versus ACE DD) x population (British: left figure, Swiss: right figure) on transcript fold change (eight hours post- exercise versus pre-exercise). Vertical bars denote 0.95 confidence intervals.

British cohort data assessed from 28S related RT-PCR data with an ANOVA.

Swiss cohort data assessed from background corrected microarray data with an ANOVA.

At the single transcript level the ACE I/Dp genotype dependent fold-change in untrained participants of only one factor, TnC, was statistically comparable between the UK and Swiss cohorts.

A subset of the Swiss population underwent an endurance training programme (Suter *et al.*, 1995; Schmutz *et al.*, 2006), and this significantly increased muscle capillarity in participants without the I-allele. Conversely, increases in intramyocellular (IMCL) lipids were inhibited in participants without the I-allele (Figure 54).

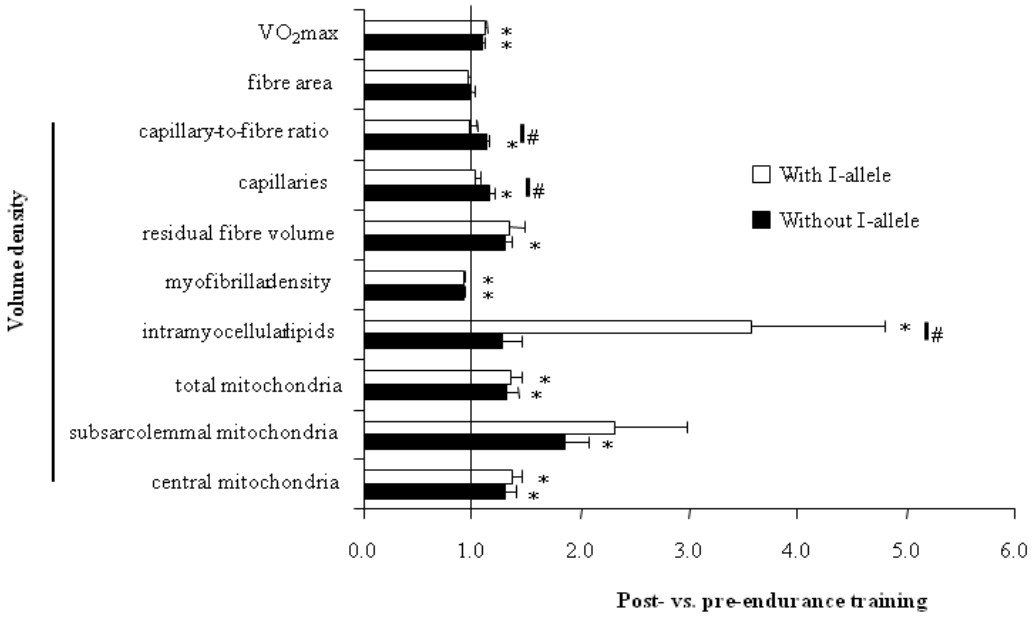

Figure 54. Local muscle changes in the Swiss cohort following an endurance training programme

Bar graph of mean +SE of fold changes in muscle parameters and VO2max in ACE I/Dp genotypes after an endurance training programme. N=10 with out I-allele, 9 with I-allele. *p<0.05 for post vs. pres changes (paired T-test). #p<0.05 for fold changes between ACE I/Dp genotypes (repeated ANOVA)

5.4. Discussion

The influence of the ACE I/Dp in two different populations (Swiss and British) were compared, at rest, at different system levels (physiological, ultra-structural and molecular). The Swiss cohort was also assessed following an endurance training regime.

Acute endurance exercise produces ACE dependent changes in substrate utilisation pathways to working muscle, of which capillary density and local lipid storage are influenced differently, following endurance training (only studied in the Swiss cohort).

The ACE I/Dp differences between the Swiss and British cohorts could possibly be explained by differences in physiological factors. The I-allele appears to be interacting differently dependent on population, as oxygen consumption and power output vary (Figure 49). This would suggest that other factors (environmental and physiological) are over-riding any influence of the ACE I/Dp. Further evidence of this is highlighted in capillary density between populations. There is a significant increase in capillary density in participants without the I-allele in the Swiss cohort, however the opposite is found (trend) in the British cohort (Figure 50). Evidence generally points towards increased capillarity in fibres with a high oxidative capacity, such as the type I fibres (Andersen & Henriksson, 1977; Laughlin & Roseguini, 2008), but ultimately capillaries will develop, regardless of fibre type, should demand necessitate (Egginton & Gaffney, 2010). Therefore you may expect to see a significant difference in capillarity between cohorts if one cohort had more type I fibres, as in the case here: the Swiss cohort displayed a trend for increased type I fibres, which became significant in the Swiss versus British cohort without the I-allele (Figure 52). Another study has investigated the association of fibre type and ACE I/Dp, but found a significant increase in type I fibres in the presence of the I-allele (Zhang *et al.*, 2003), which is the opposite of what we found. However, there was no association with ACE I/Dp and capillarity. Was this opposite effect down to differences in ethnicity, as Asian populations have been shown to have an increased frequency of the I-allele (Figure 31, (Salem & Batzer, 2009)). Although there is not a significant difference between VO_2max and ACE I/Dp, as seen is some earlier studies (Hagberg *et al.*, 2002; Zhao *et al.*, 2003), the association of increased percentage of type I fibres without the I-allele is in conflict with the majority of evidence that points towards the I-allele being associated with an endurance phenotype (Gayagay *et al.*, 1998; Montgomery *et al.*, 1998; Myerson *et al.*, 1999; Williams *et al.*, 2000; Collins *et al.*, 2004; Hruskovicova *et al.*, 2006). What is interesting are the different adaptations following an endurance training regime (Swiss cohort). In participants without the I-allele capillary density is significantly increased, whilst intramyocellular lipids (IMCL) are inhibited (Figure

54). Despite this inverted ACE I/Dp genotype dependence between Swiss and UK populations the gene expression response to exercise is preserved (Figure 53).

5.5. Conclusion

Ethnicity and the 'blurring' of differing training states (e.g. whether a population is endurance or strength trained) are possibly two key variables that contribute towards the controversy seen within the literature surround human performance and the ACE I/Dp (Woods, 2009).

In this study with male Swiss and British participants we saw evidence for a possible role of ethnicity as an explanation for differences in ACE I/Dp dependent metabolic performance between Caucasian populations. It was however interesting to note that important gene transcripts involved in angiogenesis and substrate utilisation were unaffected. The fact that capillarity and IMCL were oppositely regulated, post endurance training in Swiss participants, supports the view that the ACE I/Dp is a compensatory reaction to overcome limitations in blood flow related metabolic supply to skeletal muscle during or post exercise.

As the numbers were low in the Swiss endurance training population these conclusions must be treated with caution – as sampling errors could possibly account for the differences seen.

Together with the observations that metabolite responses were lost in trained versus untrained British participants (chapter three), here we showed that the environmental stimulus in a different population again over-rode the influences (at different system levels) of the ACE I/Dp.

Chapter 6

Epilogue: Studying the whole-organism

6. Epilogue: Studying the whole-organism

On the face of things the number of chapters presented in this thesis are fewer than would normally be expected for a PhD. However, the very nature of the research where many system levels are investigated in concert dictates that results need to be presented together. Results could have been separated (e.g. one-legged exercise, gene expression, metabolomics, physiology, capillarity), thus increasing the number of chapters, but the impact and related inter-connectivity of the different systems would have been lost – or at best harder to follow.

I would also like to present and comment on additional experiments/analysis that were performed during my research, but due to a combination of time and financial constraints I was not able to complete and include in my thesis.

Overall findings from research

Chapter 3

To really understand what is driving/limiting energy metabolism (and link it to whole-body metabolism) at the local working muscle it is vital that the muscle is maximally stressed (relatively). Traditional exercise analytical research and methodology (e.g. two-legged VO_2max) may not be appropriate to identify local muscle differences between trained and untrained populations. Although only tentatively identified, it was clear that the trained population was able to utilise both non-polar and polar metabolites much more efficiently than untrained – as highlighted by the metabolomic analysis

Chapter 4

Building on chapter 3, chapter 4 sets out to test the influence, if any, of a well studied performance genetic variation (ACE I/Dp) in these two populations (trained and untrained) at several system levels. Despite no difference in physiological measures there were significant specific gene expressional differences in ACE I/Dp untrained pre- versus post-exercise bout (Figure 47), which were lost in trained (Figure 48). There were also clear differences in the metabolite response (polar, and non-polar) in ACE I/Dp untrained pre- versus post-exercise bout, which again were lost in the

trained state (Figures 43, 44 and 45). These results highlight that the strong stimulus of exercise can over-ride any genetical inhibition that may exist – even if small.

Chapter 5

Ethnicity is often quoted as a confounding factor in interpreting results from ACE I/Dp studies. Again building on chapter 4, we showed that a different population (albeit still Caucasian – but distinctly different in origin) – Swiss, whilst having some different physiological measures (Pmax, capillary density: Figures 49 and 50 respectively) has similar gene expressional differences at rest (Figure 53). However, following an endurance exercise programme (Swiss only) capillary density and IMCL were differently regulated depending on ACE I/Dp (Figure 54).

From these three studies it is evident that there are 1) novel polar and non-polar metabolites, which are differently regulated/expressed in trained populations versus untrained, 2) ACE I/Dp gene expression and metabolite differences in untrained, which are lost in trained populations, and 3) ACE I/Dp may influence capillary supply lines and energy stores following an endurance training programme.

Additional discussion, results and methodological developments (not included in main research chapters)

The fascination and study of regular physical activity and health (or disease prevention) is not a new phenomena, see Fgure 55. But during the last century and certainly within the last 25 years there has been an ever increasing focus on why regular physical activity confers so many potentially protective health benefits (see Table 4, and Figure 21). One area of research that has generated much interest, with regard to health (and sporting performance – the two are closely linked) over the past 20 years is genetics.

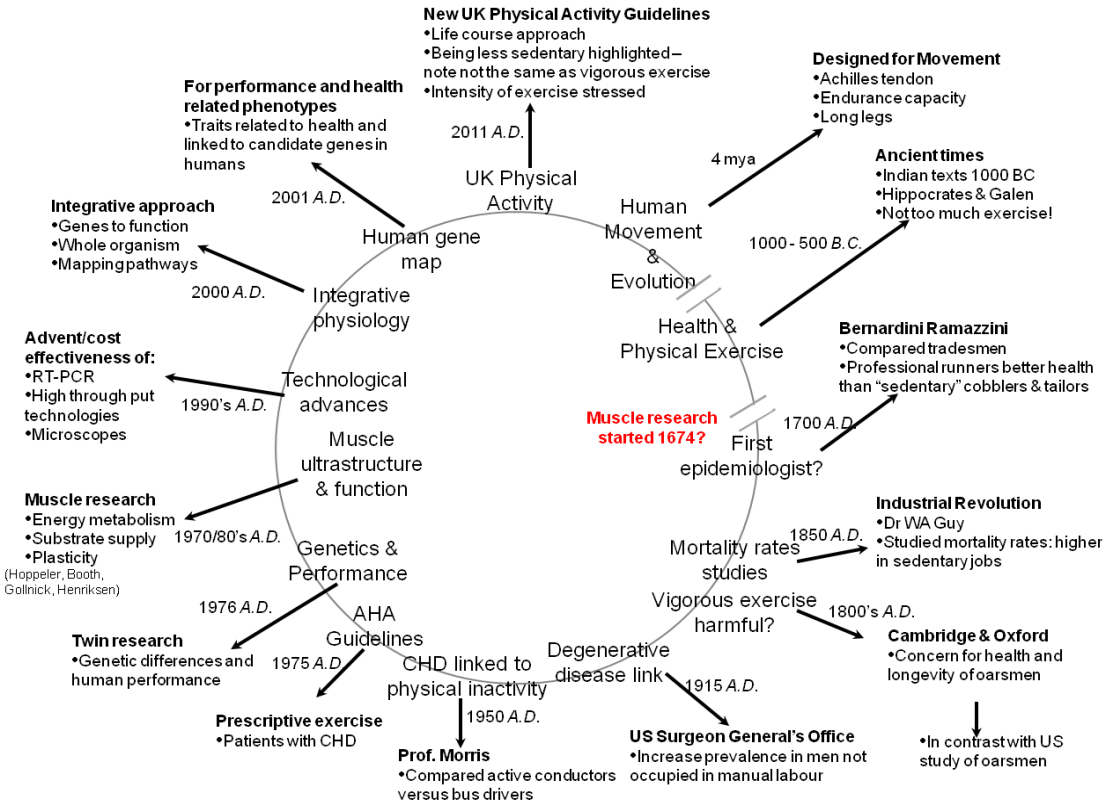

Figure 55 – Physical activity research through the ages

The aim of the figure is to provide some examples of key milestones related to physical activity research and related milestones through the ages.

If we all (humans) have the same DNA, which in turn instruct the production of the same specialist cells and organs, then why do we all not enjoy the same quality of life (QOL) and live to a ripe old age free from debilitating diseases and/or early death? The answer is a simple one: because DNA is just like the letters and words on this page, they are inert and only have a meaning (or produce a result) once read and translated (DNA---RNA---Protein). Leaving aside the monogenic diseases, such as muscular dystrophy and Huntington's disease, diseases (especially those related to lifestyle – chronic diseases related to the cardiovascular system/metabolism) are the result of an interaction between many genes and the environment over many decades – leaving aside any epigenetic influences, which is a new area only just begingin to be explored.

"There are no biological functions that rely on the coding provided by a single gene" (Noble, 2008).

Despite the avalanche of positive health benefits, the importance of physical activity in the prevention and treatment of disease is under-valued and poorly communicated. Possibly one of the fundamental reasons regarding this "lack of interest" is the paucity of concrete evidence that details exactly how it confers health benefits (Coffey & Hawley, 2007). One way for this to change is to design and perform studies that provide an insight into detailed changes/adaptations encompassing many system levels within the body – from molecules to movement. For example, studies investigating the contractile properties of single muscle fibres *in vitro* and then inferring what their findings mean at a whole body level can be misleading due to redundancy and cooperativity in biological systems. Single muscle fibres don't act in isolation; they are part of a living, moving, and complex organism.

Only by attempting to uncover pathways that show how the body, as a whole, responds and benefits at inter-connected levels can we really begin to unravel the mysteries behind the health benefits of physical activity – and this will provide strong evidence, which will be difficult to ignore. It is time to stop "looking" for associations and start identifying (or at least attempting) molecular mechanisms that result in the myriad of health benefits resulting from regular PA. This view is succinctly encapsulated by Hamilton and Booth in a review in 2000: "Because so many potential physiological and biochemical signals change during exercise, it will be an important challenge in the next century to move beyond 'correlation studies' and to identify responsible mechanisms" (Hamilton & Booth, 2000).

The integrative style of the research is illustrated in Figure 56 below:

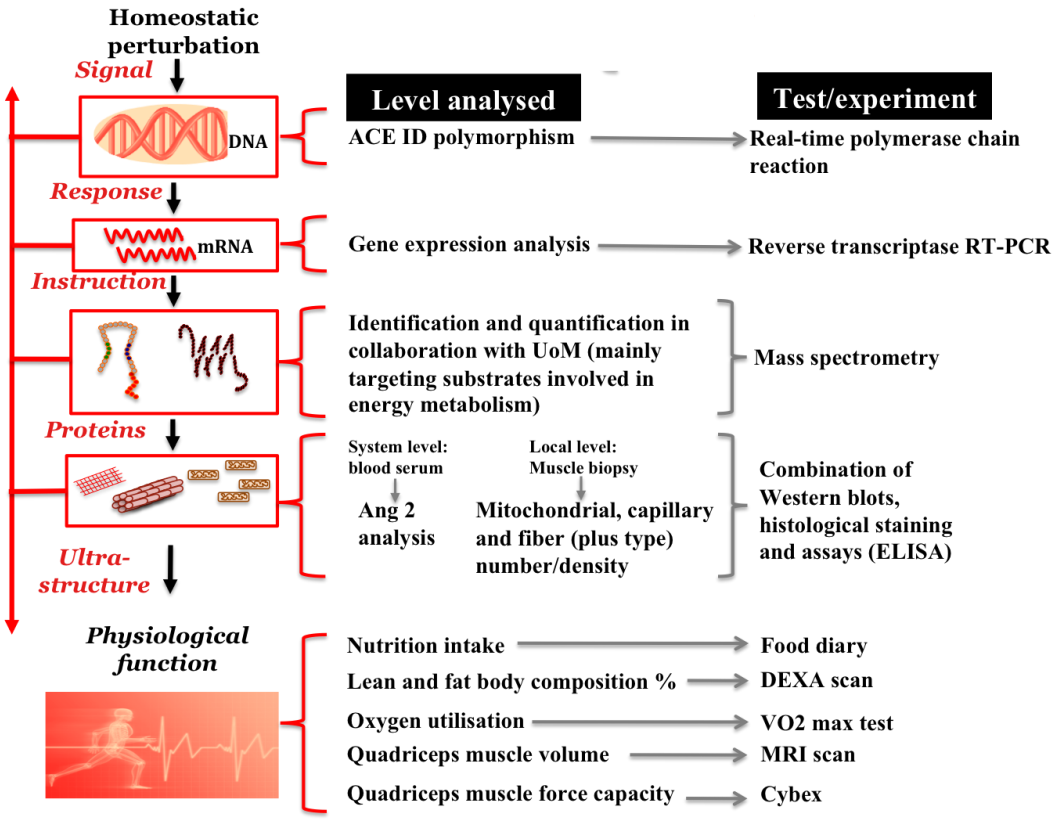

Figure 56. Integrative style of study depicting how different levels are linked

Angiotensin 2

Angiotensin 2 (Ang2) as already alluded is the key effector molecule of the RAS and it is often quoted that ACE levels have no correlation with Ang2 levels – this is something I believe to be untrue. Again whilst ACE I/Dp demonstrates a strong correlation with ACE levels (DD>ID>II) most evidence suggests this correlation does not exist with Ang2 levels. As already highlighted (chapter 1, section 1.2.3) Ang2 is a very challenging peptide to accurately measure. During my research I spent a good proportion trying to accurately quantify Ang2, with minimal success, and if there was any correlation with the ACE I/Dp and/or pre- and post-exercise changes. Whilst I was not able to successfully complete this analysis (due to cost and time constraints) I was able to set-up a reliable technique (Figure 57).

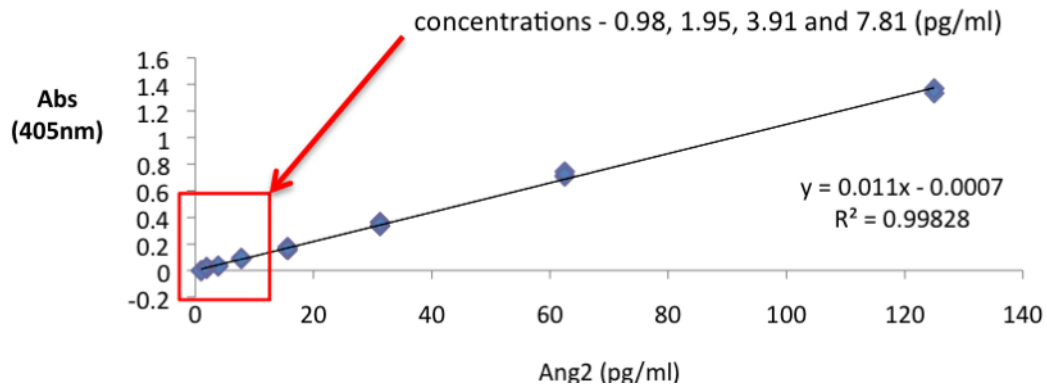

Figure 57. Standard curve – SPI BIO angiotensin 2 assay kit

Subsequent analysis on new participants from a separate study by Msc Michael Brogioli that followed up on the relationship between ACE I/Dp, Ang2 and exercise in humans has provided new evidence to support my theory that Ang2 levels depend on the ACE I/Dp (Figure 58).

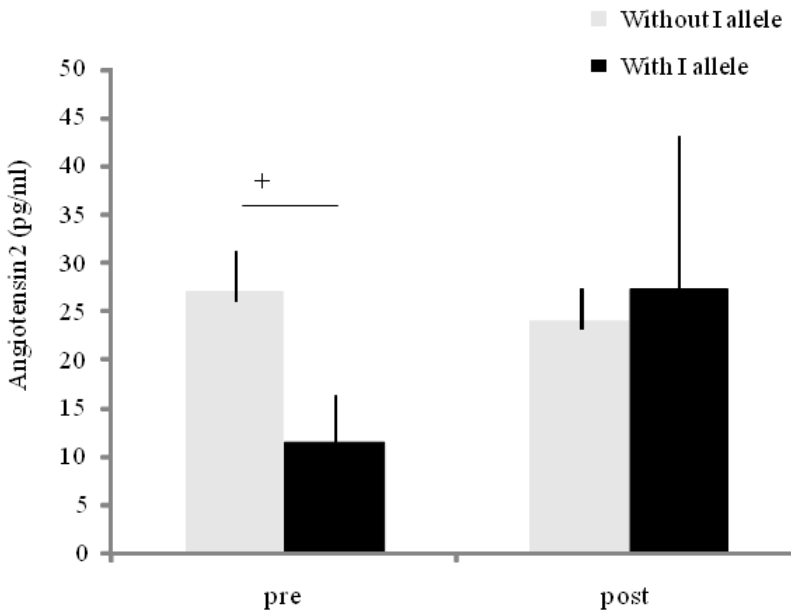

Figure 58. Angiotensin 2 levels pre- and post exercise in ACE I/Dp genotypes

n=2, without I allele; n=8 with I allele, +p=0.08. Unpublished data by MSc student Michael Brogioli.

At rest (Figure 58 illustrates) Ang2 serum levels were 134% higher (+ p=0.08) higher in ACE I/Dp genotypes without the I allele.

Methodolical problems

There are many problems in quantifying levels of Ang2 (accurately and repeatedly – constistently) below is a brief synopsis of what the more important ones were (in order of occurance) and some suggestions for overcoming:

- Mixing of all sufficient inhibitor cocktail ingredients (see SPI-BIO, Angiotensin II kit, A05880) – and ensuring they all fully dissolve
- Venous blood sampling: As the half-life of Ang2 is reported to be very short (possibly as short as 30 seconds) consistent sampling is vital as it is not always possible to withdraw 4 ml (minimum really needed to perform assay) very quickly due to variation in viscosity of participant's blood. It would also be very prudent to ensure each participant is adequately hydrated and they are in the supine position (to reduce risk of fainting)
- Another key step is the extraction protocol (extraction of Ang2 peptides) – of which the evapouration step can be troublesome. If you choose to evapourate under a stream of dry nitrogen ensure you use a large enough (deep) glass test tube and do not exert too much pressure as the fluid will likely spill over the edge. Leaving to evapourate overnight is also an option – but one that may take longer than one night
- For the final step (enzymatic reaction) which involves reading the plate (colour development) I suggest taking readings at pre-determined times following incubation, e.g. 5, 10, 20, 40, 80 to see what time produces optimal colour development and readings
- Finally, it would be ideal to get more than one sample from each participant, and one post an exercise bout (immediately) as Ang2 levels have been shown to increase with exercise. It is possible that you may not detect any Ang2 in some participants as the levels are below the sensitivity of the kit (less than 1 pg/ml) - hence taking more than one sample and/or taking a sample post exercise

Energy substrate usage

I mentioned evidence that suggested ACE inhibitors offer additional benefits other than merely reducing blood pressure (short-term benefits) (chapter 1, section 1.2.4). A key possible benefit being that ACE inhibitors increase the volume of the perfused vasculature by reducing vasoconstriction and hence increasing the ability of substrates to be metabolised by the working muscle. This has implications for people with diabetes, especially. Again, in new participants following on from my work amateur marathon runners had venous blood samples taken pre- and post-race. They were also ACE I/Dp genotyped and serum glucose was significantly elevated in marathon racers without the I allele versus those with the I allele (7.6 vs. 4.4 mM, n=4 vs. 12; p=0.0001, Student's T-test).

Whilst both the above findings come from small numbers, and the data is not accepted as a publication in a peer reviewed journal they do add some support to the fact that Ang2 levels do correlate with ACE I/Dp, and the higher levels of Ang2 (at rest) possibly offer an explanation as to why serum glucose levels are higher post race in ACE DD genotypes – they are not able to dispose of as efficiently to the contracting muscle due to a reduced vascular compartment/service area. This suggestion fits in well with the observations in chapter 5, where endurance training increased the capillary density in ACE DD genotypes only. Genotypes with the ACE insertion sequence (I) can more efficiently expand the blood vessel service area (enhancing glucose disposal) thus reducing shear stress and associated angiongenesis and new blood vessel development.

Carbohydrate and gene expression – GenMAPP

The data presented in chapter 5 included only a small number of key transcripts related to RAS and energy metabolism. The visualization of significantly changed transcripts from the larger microarray data set with GenMAPP illustrates that CHO metabolism (at a transcript level) is further increased in participants without the I allele (Figure 59). This could suggest a greater up-regulation of glucose pathways (at a transcript level) in ACE genotypes without the I-allele. It could be argued that the reduced

vascular capacity to expand blood vessels results in increased blood glucose levels (as described above). This then requires an up-regulation of glucose pathways to enable disposal of excess circulating glucose – hence related CHO transcript expression (especially glucose transporters – into muscle cells). This would be in agreement with the higher levels of glucose described above.

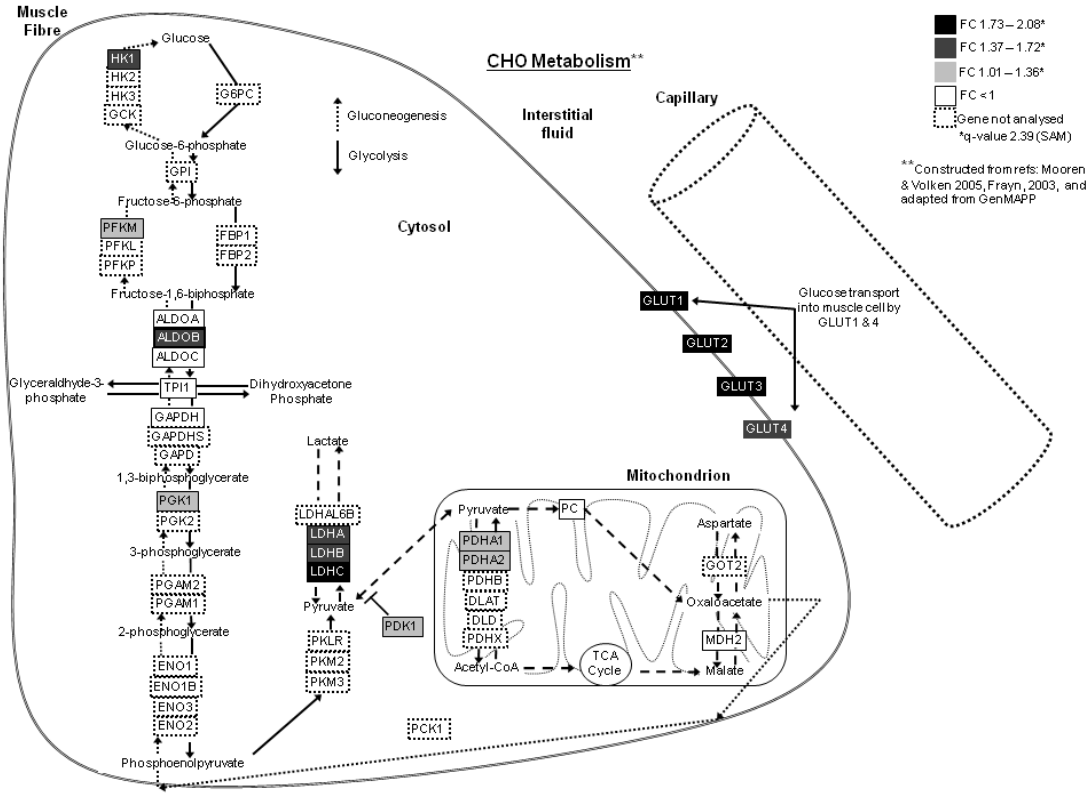

Figure 59. Gene expression of CHO metabolism transcripts: without I allele fold change difference, relative to with I allele

n=7, without I allele; n=5 with I allele

Limitations

A major limitation of my research was the combining of trained and untrained participants – a necessary concession to increase numbers. And then the resulting low numbers when trying to analyse the two cohorts separately. Originally we had hoped to accept only ACE I/Dp homozygotes (II or DD) but the low number of II's, together with the interesting results from the Swiss data, meant we accepted the heterozygote genotype too.

Another major limitation was the metabolomic data analysis. Due to no previous published work on metabolite analysis in human skelelal muscle techniques had to be set up, which proved very time consuming and problematic. After the technique was established the mass spectrum and peaks needed interpretating and unknown metabolites needed identifying – this again requires extensive resources and expertise. Some tentative non-polar metabolite identification was possible, but the equally interesting polar metabolite analysis and identification has yet to commence. Arguably the most novel and interesting part of the whole research is proved the most problematic – something that I hope will be resolved in the near future, but alas too late for inclusion in my thesis.

Concluding remarks

With such low numbers, especially when investigating a widely studied gene polymorphism, there will be much scepticism as to chance playing its part or even the experimenters own bias.

If it was not clear in the methods I would like to reiterate the fact that all tests and measures (including any potentially affected data analysis) were done blind – the main experimenter (author) did not know the ACE I/Dp genotype of any participant until the measure of molecular, cellular, anatomical and physiological parameters was complete. Any one part of the research taken in isolation (RT-PCR, microarray, MRI, DEXA, capillary density, gas measures, force, muscle metabolites, serum metabolites), especially in such a small population, would provide very little new evidence – especially regarding the potential influence of the ACE I/Dp. However, when examining the data together it becomes more interesting (challenging to

understand and explain too) and hopefully offers a new insight into why integrative style research is needed – especially when studying complex whole-organisms and trying to understand how exercise (acute and long-term) influence muscle to produce beneficial health adaptations.

Future research and devopments

The ACE I/Dp most interesting function is the possible influence it has, not on ACE levels, but on Ang2 – as this is the really interesting and effector molecule of the RAS. Therefore directing research towards identifying if there is truly a correlation between ACE I/Dp and Ang2 levels – at different time points (and within individuals) is crucial. Also, again it would be very interesting to ascertain if there are any correlations between BK levels (and or receptors – proteins not just gene expression) and the ACE I/Dp as there is very little research directed along this pathway, which is surprising given its potent vasodilory properties and the fact that many of the beneficial affects of ACEi's may be due to the potentiation of this molecule rather than the reduction of Ang2 production. Plus, BK levels my also be naturally higher in participants with the insersion sequence (ACE levels certainly are). Again keeping on the theme of identifying components of the RAS and ACE I/Dp it would be of great interest to see if it was not simply the levels per se of ACE or Ang2 that were important but the efficiency of the conversion from Ang1 to Ang2, which has been demonstrated to be different between ACE I/Dp (genotype without I allele more efficient). There is some evidence produced, but generating more would certainly be of great benefit.

Whilst it is known that lipid pathways are regulated differently in trained populations of which important proteins/enzymes have been identified, what is not known is why are some processes both beneficial and detrimental, e.g. both type II diabetics and endurance trained populations have greater IMCL stores, but this is deleterious in type II diabetics but beneficial in trained populations. Investigating one or a few metabolites at once can prove very time consuming, but analysing 10's if not 100's at once by using a metabolomic approach can be very efficient – providing systems are in place to robustly identify subsequent metabolites. This approach may

shed new light onto already existing evidence and more importantly identify new metabolites – those yet to have been explicitly implicated in energetic pathways.

Finally, using a more integrative style of research (when studying whole-organisms) would be of real benefit as it allows the usage of smaller numbers – something that is often quoted as not sufficient when investigating genetic components.

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Appendix

Appendix

Appendix 1: Ethics

Ethics Committe Reference Number: 2007.11.04

Application for Ethical Approval for the Use of Humans in Research

1. Name of responsible investigators.

David Vaughan (PhD student)
Professor Martin Flueck (Director of Studies)
Dave Tomlinson (Advisor)
Professor Joern Rittweger (GMC Registered)
Dr Hans Degens (Advisor)
Professor Marco Narici (Advisor)
Professor Roy Goodacre (Collaborator – University of Manchester)
Dr William Allwood (Collaborator – University of Manchester)

2. Title of investigation: "Systems Biology of Human Strength and Fitness" (changed later to "Integrative Phyiology of Human Aerobic Fitness and the Influence of the ACE I/Dp Genotype")

3. This is a PhD programme research project.

4. The full details have been appended.

5. Brief description (500 words maximum) and purpose of the investigation.

Metabolic diseases and frailty pose a significant health risk of which skeletal muscle (SKM) is an important component. We know that SKM is very adaptable in respect of use and disuse but that the extent of the adaptation varies between people and must reflect their underlying genetic background. However the details of the interaction between genes and environment are poorly understood.

Rationale

The response of the SKM phenotype to a stimulus such as exercise is mediated by the activation of gene-dependent signalling pathways (Booth and Neufer 2005, Flueck and Hoppeler 2003). This signalling is specific to the type of exercise and can be demonstrated as changes in mRNA over time following stimulus. These changes in gene expression are translated into protein, which results in mechanical and metabolic adaptations in SKM. Over time, and with repetition, the exercise stimulus results in a cumulative adaptation of SKM (Flueck 2006).

This project aims to demonstrate the involvement of gene-dependent pathways in an exercise-specific SKM adaptation by monitoring changes in relation to angiotensin 2 (Ang 2) activity, which is thought to be a master factor of SKM signalling for strength and fitness. The extent of the adaptation to the exercise stimulus will be contrasted in participants with high and low Ang 2 activity as a consequence of polymorphisms in the gene producing Ang 2 (ACE I/D gene polymorphism – see Sayed-Tabatabaei et al 2006 for a review). There are two-fold higher Ang2 levels in serum for ACE DD compared to ACE II genotype (Rigat *et al.,* 1990; Mondorf *et al.,* 1998; Bloem *et al.,* 1996).

General Hypothesis

Angiotensin 2 governs the balance between physiological angiogenesis (broadly, endurance adaptations) and myogenesis (mainly, strength adaptations) to an acute bout of exercise.

Specific hypotheses

In sedentary but healthy males we expect to see after an acute bout of endurance or strength exercise:

1. Participants with the ACE II genotype (less Ang2 levels) will demonstrate a pronounced up-regulation of genes associated with capillarity and muscle

mitochondrial content in comparison with those with the ACE DD as a result of endurance type exercise.

2. The ACE DD genotype (higher Ang2 levels) will show a greater myogenic response compared to participants with the ACE II genotype in response to resistance exercise.

Parts 1 and 2 of the proposed study respectively address these two hypotheses.

Experimental design: please see figure 1, which shows when each test / procedure will be performed and also how they fit together in the study as a whole.

6. (a) How will the participants be recruited?

Participants will be recruited from MMU and/or local offices/clubs/organisations by means of posters, emails, flyers and letters.

(b) Provide details on the number and type of participants likely to be involved?

We will select 9 ACE-II and 9 ACE-DD homozygotes for part one (endurance exercise acute bout), and this will be repeated in part two (strength exercise acute bout).

(c) What inclusion and exclusion criteria will be employed in the selection of participants?

The participants should be healthy, not taking any medication, non-diabetic, male, 18 – 39 years of age, BMI between 20 and 30 kg/m2. They should not undertake regular exercise (>1 hours/week). Maximal oxygen uptake (2max) should therefore not be above 50ml O2/min/kg. Eligibility for participation will be assessed by a lifestyle questionnaire adapted from Ware and Sherbourne 1992, Howley and Franks (2003) and via an online test (similar/adapted to one described by George *et al.,* 1997).

The General Health Departmental Questionnaire will be used as an additional screening tool immediately before participation of exercise. Participants will not be allowed to exercise if they have a cold, flu or fever at the time of testing, or are suffering with musculo-skeletal problems.

(d) What criteria have been employed to determine the number of participants for the study?

Assuming all participants will complete part one and part two of the study these numbers will provide nine biological replicates per given genotype and acute exercise intervention (endurance and strength). These numbers have been proven to be large enough in previous studies to detect exercise effects of genotype-dependent transcripts with the proposed molecular techniques (Schmutz *et al.*, 2006, Fluck 2006, Pilegaard *et al.*, 2000, Klossner *et al.*, 2007). This is supported by prospective power analysis on the expected transcript level changes, which indicates a prospective sample size of 6-8 to reach significance for the anticipated genotype effect (Erdfelder *et al.*, 1996).

7. Likely duration of the project and location of study

Parts one and two, should each take one year to complete (please see figure 1 for a study outline).
Briefly, the functional tests should be completed within 8 weeks. Every participant will be asked to attend for testing on 4/5 separate days (with the option of possibly another 2 separate days), each visit will last no longer than half a day, except for the final visit, which will be a whole day. It is envisaged that each participant will complete all tests within 8 weeks.
The exercise interventions will be conducted in the IRM laboratories at Alsager and/or Manchester.

Location of study**

New physiology laboratory and data collection area: DEXA scan and appropriate exercise tests.

Active lifespan laboratory: Magnetic Resonance Imaging (MRI), muscle function/strength tests.
Old physiology lab: Acute endurance and strength training bouts, anthropometric measurements (height, weight and waist circumference), blood sampling (finger prick and venous), heart rate, blood pressure, pre-test medical questionnaire completion, muscle biopsies.

The University of Manchester: Analysis of metabolites in both venous and finger prick blood and muscle biopsies in compliance with the Human Tissue Act 2004.

**Test locations will obviously change upon moving to Manchester

8. Specify the particular procedures that involve human participants.

Tests and procedures

The following tests/procedures will be performed by Mr David Vaughan (PhD student) with help from professor Martin Flueck (especially for molecular tests) and Mr Dave Tomlinson (for physiological measures). The one exception is with regard to taking muscle biopsies, which will be performed by professor Joern Rittweger.

Lifestyle questionnaire: This will be used as a screening tool, to assess potential participant's suitability for inclusion in the study, including motivation, attitude and nutrition. An online test will also be used to estimate each particip ant's VO_2max (see appendix II). See appendix I, which is an example copy of the questionnaire.

Rapid screen

Anthropometric measurements: Weight, height, BMI, waist circumference; all measured with participants wearing T-shirt and shorts.
Resting heart rate and blood pressure: Measured with a Polar heart rate monitor (or similar device – yet to be determined) and automatic blood pressure machine (exact

make and model to be determined). Participants will be sitting and rested for at least 10 minutes before measurements are taken.

Mucosal mouth swab: A cotton bud will be used to collect cells lining the inner cheek of each participant. This sample will then be analysed to determine the ACE genotype.

Based on the above results, suitable participants will be invited to take part in the study.

Dietary analysis: Each selected participant will have a thorough dietary analysis. To be performed by DV – registered dietitian.

Blood samples, venous and finger prick: 20ml venous blood samples will be collected before the dietary intervention, and at the same time points as the muscle biopsies (immediately pre-test) and at three time points after the acute exercise bout (see appendix II). The metabolites and proteins of interest are given in Tables 2 and 4. Ang2 levels will be measured in finger prick samples taken before, once during and immediately after the exercise bout. All blood sampling will be performed by a qualified phlebotomist(s) – MF, DV and DT are to attend an appropriate course on February 21st 2008.

Magnetic resonance imaging (MRI) and ultrasound: These will be used to determine the quadriceps muscle volume. Using four MRI scans, with each scan being 16cm in length, starting from the distal lateral condyle to the proximal end of the greater trochanter of the femur. Standard scanning procedures will be employed. For ultrasound see appendix II.

Maximal voluntary isometric strength: This will be determined at optimal knee angle using the Cybex.

DEXA scan: Body composition will be determined with a whole body DEXA scan.

Muscle biopsies: This procedure is required to be able to analyse transcripts, proteins, and metabolites of interest. Genes are up/down-regulated at different time points following a bout of exercise. Based on previous work by MF, 4 important time points have been identified; pre-exercise (control), 30 minutes and 3 hours post exercise (these 3 biopsies will be with the fine needle), plus a final biopsy at 8 hours, which will be taken with a conchotome – this larger sample is needed for histochemical

determination of fibre type composition and fibre size. See appendix II for a detailed explanation and reasoning. All muscle biopsies will be performed by Professor Joern Rittweger (GMC registered)

Depending on which part of the study (part 1 or 2) the participants are doing they will perform the following tests.

Part 1: Response to an endurance exercise stimulus.

One-legged cycling oxygen uptake (VO_2peak test): Participants will perform an incremental exercise test on a cycle ergometer, but using just one leg. The result will be used to set the workload for the single leg endurance-training stimulus (see below).

One-legged cycling (acute endurance stimulus): One leg exercise will be used to remove the variability that exists in two-leg exercise as a consequence of differences in cardiac output. Participants will exercise one-legged at a constant exercise intensity of 60% peak power for 25 minutes after which the power will be increased by 5W every 30 seconds until volitional exhaustion.

Two-legged cycling oxygen uptake (VO_2max test): A conventional progressive exercise test will be conducted on a cycle ergometer approximately two weeks after participants have completed the acute exercise test. These results will be used to further characterise the participants and replace the estimates made in the preliminary screen.

Part 2: Response to a resistance exercise stimulus.

Maximal single repetition (1RM): 1RM will be determined using a conventional leg extension work station. Participants will make one contraction a minute, starting at about 60% of their estimated 1RM and the load will be progressively increased until they can no longer fully extend the knee. The result will be used to set the workload for the single leg strength exercise stimulus (see below).

One-legged extension (acute strength exercise stimulus): Using the same workstation on which the 1RM was determined, participants will perform 10 repetitions, and 3 sets at 75% of their 1RM.

Molecular tests

All these techniques are selected to determine the contribution of gene-dependent pathways.

Please refer to Tables 1 – 4 for a definitive list of transcripts, proteins and metabolites to be analysed, and see appendix II.

We have already established a technique for ACE genotyping using mucosal samples (See Figure 2)

ACE genotyping: Mucosal cells will be removed from a participant's inner cheek with a cotton ear bud. ACE II and DD will be identified by real-time polymerase chain reaction (RT-PCR), using specific ACE II / DD primers. To avoid miss-genotyping the products of the initial RT-PCR will be run through another RT-PCR test with either ACE II or DD specific primers and may also be run through a gel.

Transcript profiling: This is required to identify those transcripts of interest, which may be up regulated by exercise (gene expression). A technique will be employed, which "converts" the products of gene expression (mRNA) to cDNA, by using an enzyme called reverse transcriptase. The cDNA can then be identified and quantified using RT-PCR.

Analysis of metabolites: It is necessary to measure levels of key metabolites (both locally – in the muscle, and systemically – in the blood), e.g. Ang 2, glucose, triglycerides, etc. Obviously Ang 2 is of key interest, as we expect to see significantly different levels between the two ACE genotypes. Other relevant metabolites (refer to Tables 1 to 4) will be analysed (exact number will depend on exercise test and time,

with preference being given to those of high importance) as we expect to find significant differences in response to exercise with the different ACE genotypes.

Metabolic Profiling: To be carried out by the University of Manchester. This will be an important addition to the study, which will allow key metabolites (refer to Table 4) to be identified and possibly quantified. This will provide us with further information, which will help us build up a metabolic profile, in response to the stimulus of exercise.

Previous work performed and published by MF has shown that 20mg of muscle biopsy obtained with the fine needle is sufficient for the three molecular tests described above.

Cellular diagnostics: This is required to identify fibre types, fibre-to-capillary ratio, and capillaries per muscle area. A larger "normal" size muscle biopsy is required for these molecular tests.

9. Are any novel procedures involved in this study? If so, full details must be attached.

The only procedure that is new is the fine needle muscle biopsy (details attached), which is intended to be less traumatic than the conventional biopsy technique.

10. Clearly state all substances or materials to be administered or applied. State their potential hazards, if any, and the precautions to be taken.

Muscle biopsies: Professor Joern Rittweger (GMC registered) will perform all muscle biopsy procedures. Participants will first be asked if they have ever had an adverse reaction to a local anaesthetic; if not local anaesthetic (2% Lignocaine) will be infiltrated under the skin. In the case of an adverse reaction (such as reddening or a weal mark) no further Lignocaine will be administered and the condition of the participant will be monitored for general change in colour, heart rate and blood pressure, over the following 30-60 min. In the case of a severe reaction emergency medical help will be summoned. The well being of the participants will be monitored

continuously. A defibrillation set will be available in the same building of the MMU Alsager Campus, in the extremely unlikely event of a severe reaction occurring with the anaesthetic.

Blood venous and finger prick samples: There is a small risk of infection when taking blood but every precaution will be taken to minimise this risk, with the use of sterile equipment. The blood samples will be drawn by qualified phlebotomists when taking venous samples.

11. State the degree of discomfort in terms of apprehension, pain, stress and disturbance in terms of alternation to routine.

The majority of the tests and procedures should be without stress for all participants. The exercise tests will entail a degree of effort and fatigue but this will not be excessive or prolonged and is something that the participants will be able to assess when volunteering and giving consent. Participants will be constantly monitored before, during and after all tests and will be made aware that they may withdraw from the test/study at any time. The venous blood sample may cause a very brief slight discomfort for some participants. The muscle biopsies may cause discomfort to some participants (usually the injecting of anaesthetic is the only painful part), but everything will be done to keep participants relaxed and address any concerns they may have, and to provide aftercare advice and information. Please see appendix III for a more detailed explanation.

12. State your experience or that of the supervisor or other investigators in this type of investigation.

Prof. Martin Flueck has experience in similarly invasive exercise studies with a similar population in humans (Schmutz *et al.*, 2006, Klossner *et al.*, 2007) and has expertise in 2peak testing, molecular diagnostics and biochemical tests. Professor Joern Rittweger is experienced in performing invasive muscle biopsies, including fine needle biopsies. Prof. Roy Goodacre and Dr William Allwood are experts in the field of metabolomics (Hollywood *et al.*, 2006). Dr Hans Degens has experience of muscle histochemistry

and capillary measures. Prof. Marco Narici has experience in strength training design and analysis. Mr Dave Tomlinson has experience of physiological and functional measures and Mr David Vaughan (registered dietitian) has experience of nutrition, diet analysis, questionnaire design and lifestyle assessment.

13. How and where is the data to be stored?

Will the data be securely stored? Where?

All paper hard copies of questionnaires and tests will be kept locked away in a filing cabinet.

All electronic data will be stored on a server and/or on a mobile hardware device (to be securely locked away – e.g. external hardware drive and USB stick), in a password protected file. The participants' names will be coded in compliance with the Data Protection Act 1998. We will only work with the 'blinded' data (i.e. will obviously know the names of participants but will not know their exact genotype – only that they are either homozygous II or DD) and save the 'key' relating code to names in a safe place. To achieve this, a person external to the main investigations will be responsible for coding the data (Professor David Jones).

Will information which could identify participants be coded? Yes
Will the data be destroyed at the end of the study? No.

The data will be archived in compliance with the guidelines for good laboratory practice. Files of raw data shall be kept at a secure place for approximately 5-10 years after the review of planned publications has been completed. This will enable any future meta-analysis, should there be any relevant need.

Appendix II

Lifestyle questionnaire: Each participant will be asked to complete a detailed, validated questionnaire (adapted from Ware and Sherbourne 1992, and Howley and

Franks 2003) to assess their anthropometrics, lifestyle, physical activity/exercise levels, current and past medical health, general quality of life and dietary habits, which will allow us to assess the participants suitability for study inclusion. The theory behind the completion of this questionnaire is to try (as much as possible) and select participants who will be best able to complete the whole study. This questionnaire may prove unsuccessful therefore, we may modify/simply scrap it if it fails to identify enough suitable participants and simply pick those that meet the minimum requirements (are male, aged 18 – 39 years of age and are sedentary), providing there are no health issues.

Non-exercise test for VO_2max: A 'dry' assessment of general fitness will be used to estimate the participants VO_2max. This will be achieved via online access to http://www.brianmac.co.uk/vo2maxnd.htm. We will perform one/two VO_2max tests (approximately two weeks after the participants have completed the exercise test). This is to reduce the risk that the VO_2max test may actually reduce the affect of the single acute exercise test and/or give them an extra push or incentive to become more physically active between starting the study and taking the exercise test, we are trying to minimize any "noise".

Anthropometric measures:

Height, weight, BMI (body mass index), WHR (waist-to-hip ratio) measurement to compare against BMI. These are all very simple, straight forward measurements that are non-invasive.

Resting heart rate and blood pressure: Sphygmomanometry will be used for the static measure of mean arterial pressure. A suitable automated blood pressure measuring device (yet to be determined), validated and recommended by the British Hypertension Society (O'Brien *et al.,* 2001(a)) will be used. Protocol for use will be followed as recommended by O'Brien *et al.*, 2001(b).

Mucosal mouth swab: There must be no cuts or ulcers in mouth, the mouth must be cleaned (e.g. swilled out with water – several times) immediately before sample

collection. Participants will also be asked to refrain from drinking (other that water) and eating one-hour before sample collection. Sample collection is simple and pain free, it involves collection of cells from the mucosal lining inside of the cheeks by simply twirling/rubbing an ear bud against the inner cheek wall. This technique has already proved successful, as we have recently identified the different polymorphisms of the ACE insertion/deletion sequence by using this method (refer to Figure 2).

Dietary analysis: Each subject will have their dietary habits analysed, which may include an interview, analysis of a four-day food diary and/or analysis of photographs of all food consumed, if they have a mobile phone with suitable photographic capabilities (jpeg-pictures at 640 x 480 pixel resolution, with a file size of 62 kb). Participants may also be supplied with electronic scales and be asked to keep all food wrapping if they have do not possess a suitable phone/camera. All dietary analysis is to be performed by DV.

Blood samples, venous and finger prick: Following a 12 hour overnight fast, one 20 ml blood sample will be drawn from a forearm vein (in the first meeting – see figure 1). Blood samples will also be taken during the acute exercise bout; pre-test (and possibly during the exercise bout – finger prick only) and post test, at the same time points as the muscle biopsies. All blood samples will be collected into pre-chilled, pre-marked (for sample identification) tubes. All venous blood samples will be taken by a qualified phlebotomist. Finger-prick blood samples will be taken using disposable lancets. An electronic blood-monitoring device will also be used to measure certain metabolites (test strips and capability permitting – refer to Table 2). All blood samples will be analysed for small key metabolites by colleagues at Manchester University (refer to Table 4).

Magnetic resonance imaging (MRI) and Ultrasound: These will be used to measure muscle volume and architecture of the quadriceps femoris muscle group (VL, VI, VM and RF), at rest, which will allow calculation of physiological cross sectional area (PCSA). The volume will be calculated from three to four scans of the thigh (each scanning region will be 16cm in length). Scans will be carried out using a 0.2 T magnetic resonance imaging (MRI) scanner (Esaote Biomedica, Genoa, Italy)

following the Manchester Metropolitan University safety guidelines from the safety document for ESAOTE 0.2 Tesla MRI system E-Scan. Participants will be scanned in the supine position.

Muscle architecture of the quadriceps femoris muscle group will be analyzed using real-time Bmode ultrasonography (ATL-HDI 3000, Bothwell, USA). A 7.5 MHz linear array probe will be placed in the central region corresponding with 50 % of each muscle length, over the mid-sagittal axis of the muscle-tendon complex after identifying and marking the vertical axis of the muscle. An eco-absorptive marker will be placed over the centre of the measurement site for each individual muscle to provide a reference marker for muscle architecture measurements. Probe placements will be recorded on an acetate paper sheet to ensure the repeatability of the measurement site in the subsequent testing session if needed.

Maximal voluntary strength- static contractions: Measurements of maximum voluntary contraction torque (MVCs) will be made using a Cybex dynamometer (Cybex Norm, Cybex International, New York, NY, USA). Seat adjustments, body position and joint angle will be optimised for each participant and kept constant throughout the trial. At least three MVCs, each lasting 2-4 seconds, will be performed with a rest-interval of 1 min. This will be carried out twice, once for familiarization and again for actual measurements. Both legs will be tested. EMG signals from the hamstrings will be recorded with bipolar silver chloride surface electrodes (20 mm interelectrode distance). EMG activity will also be recorded from the Biceps Femoris muscle during the MVC. Before placing the EMG electrodes, skin impedance will be reduced below 5 kHz by standard preparation including shaving, gentle abrasion and cleaning with an alcohol-based tissue pad. The electrodes will be placed along the sagittal axis over the muscle belly, with the reference electrodes on the tibial condyle. Raw EMG signal will be rectified and averaged (Acknowledge, Biopac System Inc.). To convert the EMG measures of the hamstrings into a torque, participants will be asked to make a maximal knee flexion during which the EMG will be recorded.

DEXA scan: This measure will be performed to assess body fat composition. Body composition in terms of percent body fat, lean body mass and bone mineral content can be assessed by dual energy x-ray absorptiometry (DEXA) with a Lunar Prodigy

Advance (GE Healthcare, Waukesha, Wisconsin, USA). This shall be done in whole body mode. The scanning takes approximately five minutes and is without any inconvenience. However, there is a theoretical risk involved by the radiation imposed. In reality, that risk is rather low, as total body equivalent dosage is expected to be 0.4 microSievert. This could be compared to the background radiation of more than 2000 microSievert per year, or to the additional radiation of 10 microSievert per hour of air flight.

One-legged cycling oxygen uptake test (VO_2peak test): Participants will complete two incremental exercise tests (VO_2peak tests) to the limit of tolerance for one-legged cycling exercise (Davies & Sargeant 1975) on the electrically braked cycle ergometer. These two tests will be separated by at least 72 hours. Following a two minute warm-up period at 40W, cycling with one leg, the external work rate will be increased by a further 10W every minute. A pedal frequency of 70 rev.min-1 will be maintained throughout the exercise period. The test will be terminated when the pedal rate falls consistently below 60 rev.min-1. Pulmonary gas exchange will be measured breath-by-breath (Cosmed K4b2, Italy) and VO_2peak and peak power will be determined using standard techniques. Heart rate will be monitored throughout with a Polar heart rate monitor (exact device(s) yet to be determined, probably - Polar Electro, Kempele, Finland). Saddle and handlebar positions will be adjusted for each participant and the positions recorded to allow accurate replication of the test conditions in the acute endurance test (see below).
Immediately following termination of the test, the participants will be encouraged to perform an active recovery by cycling at a very low external work load using two legs. This should reduce sensations of local muscle fatigue and light-headedness.

One-legged cycling (acute endurance test): Each participant will have their peak power calculated from the one-legged cycling oxygen uptake tests (described above) and will exercise at 60% this peak power for 25 minutes, followed by an external work rate increase of 5W every 30 seconds until volitional exhaustion. Staessen et al (1987) demonstrated that exercise at 60% of peak aerobic two-leg exercise capacity elevates Ang 2 levels in serum by ca. 60%. Pulmonary gas exchange will be measured breath-by-breath (Oxycon alpha, Jaeger, Germany) and VO_2peak and peak power will be

determined using standard techniques. Heart rate will be monitored throughout with a suitable device (possibly a Polar Accurex plus monitor). Saddle and handlebar positions will be set to previous settings, as recorded in earlier tests.

Maximal single repetition (1RM): The dynamic strength of the knee flexor muscles will be assessed by determining the maximum weight with which a participant can perform one unilateral standard leg extension or leg press exercise (Technogym, Gambettola, Italy). The 1RM protocol will be modified from Baechle et al. (2000) and will commence with a warm-up of three repetitions against weights at ~50% of the participant's predicted 1RM. After 60 seconds of rest, the weight will be increased to 90% of the estimated 1RM. Following 60 seconds of rest, the weight will be increased to 100% for a 1RM attempt. Thereafter, successful attempts will be followed by further addition of weights to determine the 1RM in three to five attempts. This procedure will be performed prior to the one-legged knee extension exercise session (detailed below).

One-legged knee extension: Participants will be asked to perform, on a weight-lifting machine (Technogym), 3 sets of 10 knee extensions at a load corresponding to 75% of the 1RM. The amount of work (Joules) performed in each lift will be equal to m.g.h, where m=lifted mass (kg), g=acceleration of gravity (9.81 m.s-2) and h= vertical displacement (metre) of the mass measured with a linear position cable potentiometer. Hence the mechanical power developed in each lift will be equal to: (m.g.h) / t, where t = time in second. Therefore, the instantaneous power output will be given by m.g.dh/dt. A rest of 60 s will separate each series of 10 lifts.

Two-legged cycling oxygen uptake (VO_2max test): Each participant will complete two incremental exercise tests to the limit of tolerance for two-legged exercise on an electrically braked cycle ergometer approximately two weeks after the acute exercise test. Each test will be separated by at least 24 hours. Following a four-minute warm-up period at 40W, the external work rate will be increased by a further 20W every minute until volitional exhaustion. A pedal frequency of 80 rev.min-1 will be maintained throughout the exercise period. The test will be terminated when the pedal rate falls consistently below 70 rev.min-1. Pulmonary gas exchange will be measured

breath-by-breath (Cosmed K4b2, Italy) and the gas exchange threshold and 2max/peak will be determined using standard techniques. Heart rate will be monitored throughout with a suitable device (possibly a Polar Accurex plus monitor). Saddle and handlebar positions will be adjusted for each participant and the positions recorded to allow accurate replication in each test. Immediately following termination of the test, the participants will be encouraged perform an active recovery by cycling at a very low external workload. This should reduce sensations of local muscle fatigue and light-headedness.

Fine-needle biopsy procedure: A very small (~ 20 mg) biopsy will be taken from the VL muscle of the quadriceps between the upper third and the lower two thirds of the distance between the knee and the head of the femur. The micro-biopsy will be performed with a spring-loaded and reusable instrument; the Onecut Disposable biopsy (14 Gauge x 150 mm, product code ONE-141502, UK BIOPSY Ltd, Great Britain). Participants will first be asked if they have ever had an adverse reaction to a local anaesthetic; if not local anaesthetic (2% Lignocaine) will be infiltrated under the skin. After the skin has been shaved and sterilized an incision in the skin will be made to reduce resistance and allow successful sampling with the needle from the VL muscle. A muscle sample will then be obtained by the activation of a trigger button, which unloads a spring and activates the needle to collect a piece of muscle. We estimate that 20mg of muscle sample will be sufficient for all molecular and biochemical tests, and in a study by Hayot *et al.*, 2005, using the fine-needle biopsy technique, muscle sample average weight was approximately 32mg. The muscle sample will be placed on weighing boat, rapidly weighed and immediately frozen in liquid nitrogen and stored at – 80^{o}C for later analysis. Once any bleeding has stopped the tiny needle hole will be covered and closed with Steristrip(S), a sterile plaster and appropriate dressing. Professor Joern Rittweger will perform all muscle biopsies.

Muscle biopsies will be later analysed (to identify and quantify transcripts and proteins), using reverse transcription, real-time polymerase chain reaction, gel-electrophoresis and Western blot, and possibly other techniques as required.

"Normal" muscle biopsy: The procedure for the last muscle biopsy will be exactly as described above (fine-needle), with the difference being that a chonchotome (instead of the fine needle) will be used to extract a larger sample (~ 200mg). This method is currently the preferred method of muscle biopsy sampling at MMU, and Prof. Joern Rettweger has extensive experience in this.

Muscle biopsy: The time points are based upon published human work of MF to allow differentiation of the ACE- and exercise-dependent course of the different waves of the molecular response (Booth and Neufer 2005; Schmutz *et al.*, 2006, and refer to Figure 3 below). Fine needle biopsies are chosen (for the first 3 samples) since they provide sufficient biological material (see Schmutz et al 2006; Klossner *et al.*, 2006) but are less invasive than the conchotome for repeated sampling.

Pre: This measure is the reference for the determination of the molecular exercise response of each individual from the repeated biopsy measures after the single exercise. To be taken from the non-exercised leg.

30-60 minutes: At this time mechanical signalling and the first wave of downstream gene expression (c-jun pathway) is increased (Puntschart *et al.*, 1998, Klossner *et al.*, 2007). These changes are under control of ANG2-FAK pathway (Mehta and Griendling 2007), which evolves a major topic in control of the muscle phenotype (Fluck *et al.*, 2007).

3 hours: This is the earliest time when myogenic factor expression is increased after strength type exercise (Klossner *et al.*, 2007).

8 hours: Metabolic transcript expression is increased after endurance type concentric exercise (Schmutz *et al.*, 2006). This time-point allows us to distinguish from the response after eccentric type exercise. A conchotome sample will be collected to allow the orientation of muscle fibres for capillary density measures per fibre area.

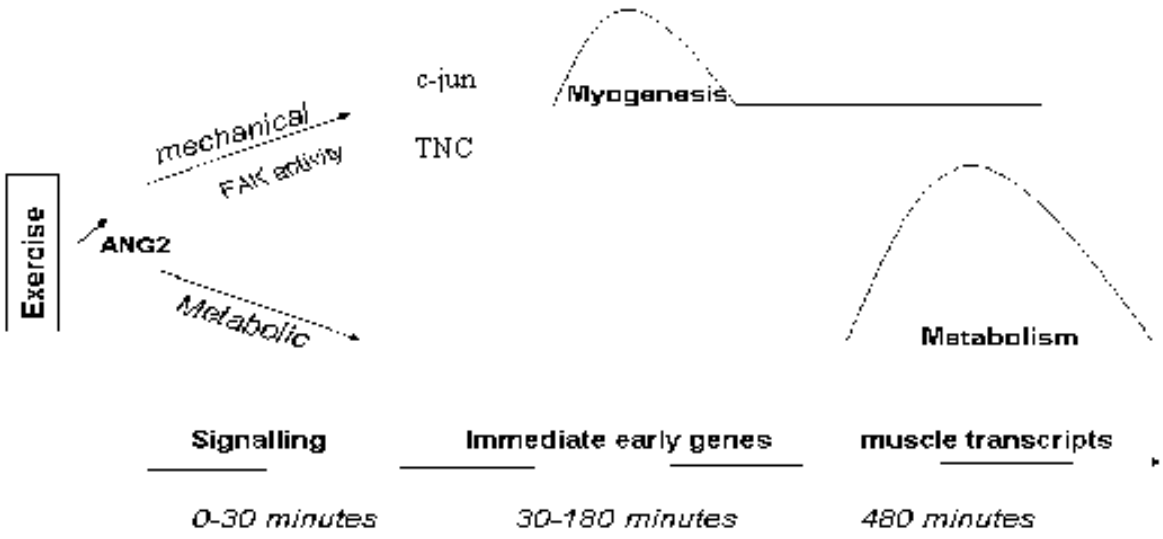

Figure 1 (Ethics)

Molecular tests

Please refer to Tables 1 to 4 for a definitive list of transcripts and metabolites to be analysed (time permitting)

ACE genotyping: This procedure has been adapted from published protocols (please refer to Badenhop *et al.,* 1995 and Evans *et al.,* 1994, plus Figure 2 for further details). We have already established a successful technique from mucosal samples.

Transcript profiling: Total RNA will be isolated from 25 µm cryosections of the muscle biopsies and quantified as described previously (Schmutz, Fluck 2006). 1 µg RNA will be reverse transcribed and subjected to real-time polymerase chain reaction (RT-PCR) for selected transcripts. cDNA will be quantified. Dependent on the primers, reactions will be run with Sybr Green or Taqman master mix (Applied Biosystems) on a Biorad DNA machine controlled by the MJ Opticon Monitor software (Biorad). 40 standard cycles (1 min 95ºC, 1 min 60ºC) will be performed after a hot start on same amounts of RNA. Primers will be used as established (Zoll *et al.,* 2006) or designed with primer express software (Applied Biosystems) and synthesized at Sigma, Genosys. RNAs to be compared will be run in a design allowing referencing. Signals will be expressed per input amount of cDNA. Alternatively,

signals will be related to the internal reference 18S RNA which will be run as a multiplex reaction in the same well (attention saturation effects).

Analysis of metabolites: This methodology will be established from existing protocols for selected metabolites in blood and muscle samples. For finger-prick blood samples the tests will be established for angiotensin 2, glucose and triglycerides. Angiotensin 2 concentration will be measured with a sandwich assay (Fildes *et al.,* 2005). In brief, 100 μL of blood will be collected with a large finger-prick (or if this isn't possible then a venous blood sample will be taken). 11 μL of 0.1 M sodium citrate will rapidly be added as an anticoagulant (or alternatively heparanised tubes may be used). Samples will then be centrifuged at 2000 rpm for 10 minutes. The plasma will then be removed and assayed with the Sandwich Elisa (AssayMax Human Angiotensin II ELISA Kit). The undiluted sample will be stored at -20ºC or below for up to 3 months. Glucose and (bound) triglycerides will be measured from the collected sample with a specific instrument and test strips.

Optionally, the suitability of test-strips for the measurement of diacylglycerol, lactate, Beta-hydroxy-butyrate, pyruvate and acetoacetate will be evaluated. Also optionally, ACE activity will be assayed with a commercial spectrophotometric ACE assay system (SIGMA Diagnostics®, St Louis, USA). In this regard see Barceló et al 2001.

The suitability of the above techniques for angiotensin 2, glucose (glycogen) and triglycerides on muscle extracts will be validated. The measure of triglycerides and glycogen is important and might have to be achieved via other means (see Essén-Gustavsson and Tesch, 1990).

Metabolomic profiling: This analysis will request additional ethical approval by the University of Manchester as it is to be carried out on biopsy samples at the metabolomic unit of Professor Royston Goodacre by Dr. William Allwood. In brief, 20 mg of frozen muscle sample will be cryo-sectioned at 25 μm and transported to Manchester. Muscle components will be extracted with a modified Fiehn protocol and separated for polar and non-polar metabolites. For an overview see Hollywood et al 2006.

Cellular diagnostics: Microanatomy of muscle: Cross-sections will be prepared at 12 μm from the biopsies and stained with monoclonal antibodies specific for fast (My-32) or slow type myosin heavy chain (MAB1628) (Schmutz et al 2006) with the modification that fluorescent antibodies will be used (Molecular probes). In addition, capillaries will be double stained with rabbit anti Ulex europaeus agglutinin I lectin on the same section, essentially as described by Qu *et al.,* 1997.

Fibres will be typed according to the expression of fast and slow myosin types into slow, fast and hybrid population. Staining will be visualized using a confocal microscope (Leica SP5). Signals will be recorded from different, non-overlapping fields for evaluation of mean and fibre type-specific 'capillary-to-fibre ratio' and 'capillaries per muscle area'. Percentage and mean cross-sectional area of each fibre type will be determined by stereological counting of all fibres from the assembled microscopic field of the biopsy cross-sections.

Appendix III

Participants will encounter disruption to their normal routine caused by the scheduled testing and procedures. The one-legged and two legged VO_2peak tests, and strength and endurance tests are hard work and some participants may feel light-headed after these tests. A sufficient low intensity cycling cool-down period will be implemented to avoid this as much as possible (with the VO_2peak test), and a chair will also be available should the participant feel light-headed following the cool-down period.

The majority of testing procedures should be without stress for the participants. Taking a venous blood sample is a minor procedure with minimal discomfort and participants will be supine to minimise any danger of fainting.

The procedures for muscle imaging (MRI, ultrasound and DEXA scan) are without sensation and are without known health-risks.

The muscle biopsies are not particularly painful; however, it is an invasive procedure which some people find unpleasant. The most painful part is the initial injection of local anaesthetic, which stings but this lasts only a few moments and after that the sensations are mainly ones of pressure as the fine-needle is inserted through the skin and the muscle sample removed. There may be some bleeding and bruising around the biopsy site, which may remain tender for a day or so. The sensation is

similar to that of a bruise. The wound has to be kept dry and clean for about four days while the skin heals but there have been no reports of any complications. Should complications occur, such as signs of infection, the participant will be advised to seek immediate medical assistance from his local general practitioner or the nearest hospital and to keep us informed.

Appendix IV

Informed Consent Form (to be retained by the investigator and participant)
Participant:

Name: Sex: Male

Date of Birth:

Principal Investigators: Prof Martin Flueck and Mr David Vaughan

Collaborators: Prof Joern Rittweger, Prof Marco Narici, Mr Dave Tomlinson, Dr Hans Degens, Prof Roy Goodacre and Dr William Allwood

Ethics Committee Approval Number:

Project Title: "Systems Biology of Human Strength and Fitness"

Purpose of study and brief description of procedures.
(Not a legal explanation but a simple statement)

Athletic ability is related to metabolic fitness, which assessed during exercise is a good predictor of health and morbidity as it is a complete task that exposes the functionality of the whole body system.

We will be taking a holistic approach, by looking at the structure of skeletal muscle (including fat, types of muscle fibres, cellular components and capillary network) and how it responds to exercise – both endurance and strength.

This will entail screening for a known, possible “fitness gene” that may play a role in how skeletal muscle responds to exercise.

Why do some people and families seem more prone to particular diseases, e.g. heart disease, diabetes, certain cancers, osteoporosis, etc? Whilst environment factors undoubtedly play a role, the genes we have certainly impact greatly upon our chances of leading a healthy life. This and future research will hopefully influence clinical treatment, by uncovering the signalling pathways that are up-regulated by exercise. Ultimately, we would like to influence how people are treated.

It is quite feasible that in the future people would receive personalised health plans, to not only prevent disease, but to also help recovery from them. This would include specific exercises and nutrition based on their genotype. This type of treatment would be a more natural approach than the current situation where medication is widely used to help manage diseases, rather than addressing the root of the problem.

If you decide to take part you will receive invaluable information and feedback on a whole host of import parameters such as, breath-by-breath gas analysis (related to fitness), body composition (including body fat percentage of different fibre types), blood analysis of factors related to cardiovascular (heart disease) health, and a detailed analysis of your diet together with expert advice on how to improve it (from a registered dietitian), should you want to.

The study will be conducted in several stages:

You are free to refuse any test and withdraw from the study at anytime, for no reason, even after signing up.

Explanation of tests and procedures

There will be a minimum of 5 visits, of which 4 will last up to ½ a day (4 hours), one visit lasting a whole day (9 – 10 hours).

Lifestyle questionnaire: This is used as a screening tool to assess potential participants' suitability for the study, we do not know the response we are going to get, and if it's large this will help us to be more selective. This will take approximately 15 minutes.

Mucosa sample: A small sample of cells from your inner cheek will be collected with an ear bud. This is completely painless. Please don't eat or drink (except water) any food an hour before and your mouth should also be free from any gum disease, ulcers and cuts. This will take approximately 5 minutes to complete.

Anthropometric measures: Height, weight, resting heart rate and blood pressure (both analysed using a Sphygmomanometer). These will take approximately 45 minutes to complete.

Dietary assessment: You will be asked to either fill in a food diary for 4 days (including the weekend) and/or (if you have a suitable mobile) take pictures of everything you eat and drink. You will be given guidance on portion sizes and how to record the information if you choose to complete a food diary. Further clarification may also be sought by means of an informal chat regarding your dietary intake. After analysis of your diet a suitable personalised nutrition plan will be formulated for you, which will be based on healthy eating guidelines. You will be asked to follow this for 3 weeks. You won't be asked to eat any foods that you don't normally eat, but you will be asked to adhere as strictly as possible to your personalised nutrition plan. This is a very important part of the study, basically we are trying to "normalise" all participants nutritional intake so that any change that we find after the exercise tests (in the blood and muscle) can be attributed to the exercise and not to some component of your diet, e.g. certain foods and compounds within them are known to increase/decrease gene expression.

Assessment of muscle size: The size of your quadriceps (thigh) muscles will be determined using magnetic resonance imaging (MRI) and ultrasound. This is a painless method of producing high quality images of tissues and all you have to do is

lie still for 30 minutes (not continuously – in bouts of approximately 5 minute periods). The only constraint is that you should have no metal on, or inside you, as the machine uses high magnetic fields to generate the images. We will check that you do not have any metal before you enter the machine. There is no pain or discomfort involved and the measurement takes about 45 minutes in total including setting up.

Maximal strength test: Maximal voluntary strength of the Vastus Lateralis (VL) muscle (right and left leg) will be made using a piece of gym equipment that looks like a leg (hamstring) curl machine – called a Cybex dynamometer. This entails you contracting your thigh/quadriceps muscle as hard as you can for approximately three seconds. This will be performed three times on each leg. This test is completely painless (apart from your effort when contracting your muscle). These measurements will take approximately 60 minutes, most of which involves the actual setting up of the equipment to an individual's dimensions.

DEXA scan: We will be measuring substances involved in exercise metabolism, and fat plays an important role in this, hence we want to accurately measure body fat percentage. Body composition in terms of percent body fat, lean body mass and bone mineral content can be assessed by dual energy x-ray absorptiometry (DEXA). The amount of radiation emitted (and therefore risk) is very low – as the total body equivalent dosage is approximately 50 times less than a 2 ½ hour air flight. There is no pain or discomfort involved and the measurement takes about 15 minutes in total, including setting up.

One-legged cycling oxygen uptake (VO_2peak test): This involves sitting on an exercise bike and cycling with one leg. You will start off with the resistance set relatively low and then every 30 seconds the resistance will be increased until you cannot cycle any more. While you are doing this we would like you to breath through a mouthpiece so we can measure the amount of oxygen you are using. We will also measure your heart rate using a monitor fitted around your chest. We would like to do this test twice. The exercise itself takes about 30 - 40 minutes but you should allow about 75 minutes in total for setting up the test, adjusting the cycle for you and for

warming up and cooling down afterwards. The test is not unpleasant but for the last part you will be working hard and will feel hot and breathless.

One-legged cycling (acute endurance test): This test is very similar to the above test except a bit longer. Based on the results from the above test you will exercise at a constant intensity (using one-leg) for 25 minutes and then the resistance will be increased every 30 seconds until you cannot cycle any more. While you are doing this we would like you to breath through a mouthpiece so we can measure the amount of oxygen you are using. We will also measure your heart rate using a monitor fitted around your chest. Before starting this test you will have a cannula inserted into a vein in your arm (to enable blood sampling at various time points) and a small sample of muscle taken from your non-exercising leg. Upon finishing the test, you will then have two very small muscle samples and one small sample taken from your exercised leg at three separate time points – 30 minutes, 3 hours and 8 hours post test (together with blood samples at similar time points). Please see below for a more detailed description of these procedures. You will be required to stay on the campus during this time. We will provide entertainment (you will be able to watch a DVD, surf the web, listen to music, watch TV etc) and food/fluid during this period. The test is not unpleasant but for the last part you will be working hard and will feel hot and breathless.

Two-legged cycling oxygen uptake (VO_2max test): This involves sitting on an exercise bike and cycling with two legs. You will start off with the resistance set relatively low and then every minute the resistance will be increased until you cannot cycle any more. While you are doing this we would like you to breath through a mouthpiece so we can measure the amount of oxygen you are using. We will also measure your heart rate using a monitor fitted around your chest. We would like to do this test twice. The exercise itself takes about 30 - 40 minutes but you should allow about 75 minutes in total for setting up the test, adjusting the cycle for you and for warming up and cooling down afterwards. The test is not unpleasant but for the last part you will be working hard and will feel hot and breathless.

The following two tests (maximal single repetition and one-legged knee extension) are not applicable to participants in part one of the study.

Maximal single repetition (1RM): This test is for calculating the maximum weight you can lift (by one-legged knee extension) once. We need to know this to work out what weight you will lift in the acute strength exercise test (see below). The test is not unpleasant, but you may feel breathless and your leg will ache towards the end of each set. You will not be performing this test if you are participating in part one of the study, as we are looking at the affect of endurance exercise.

One-legged knee extension (acute strength test): This test is performed sitting down, with your back straight and one foot "hooked" under a padded lever. With you leg in a bent position, you will contract your quadriceps muscle and pull your leg towards and up. This will be performed against resistance (weights). You will be asked to lift 75% of your 1RM weight 10 times, have a 1 minute rest then repeat this twice more. The test is not unpleasant, but you may feel breathless and your leg will ache towards the end of each set. Before starting this test you will have a small sample of muscle taken from your non-exercising leg. Upon finishing the test, you will then have two small muscle samples and one "normal" size sample taken from your exercised leg at three separate time points – 30 minutes, 3 hours and 8 hours post test (together with blood samples at similar time points). Please see below for a more detailed description of these procedures. You will be required to stay on the campus during this time. We will provide entertainment (you will be able to watch a DVD, surf the web, listen to music, watch TV etc) and food/fluid during this period. The test is not unpleasant but your leg will ache and you may feel hot and breathless towards the end of each set.

Blood samples (finger prick and venous): Physiological and metabolic changes occur after exercise, which mainly occur in the muscle. We would like to analyse and compare metabolites produced in the blood after exercise with changes in muscle samples. To do this we need blood samples. Where we can we will use finger prick blood samples, otherwise we will use a vein in your forearm. A small blood sample (5ml) will be taken from a vein in your forearm (at several different time points during

the acute exercise bout). This is not a painful procedure but some people are a little squeamish about blood and may faint. With the exception of the blood sample taken whilst exercising you will be seated whilst the blood samples are collected. This is because sometimes people become a little squeamish and may faint. Occasionally there is a little bruising. Qualified personnel will take the blood sample. Pressure will be applied for approximately one-minute and a small plaster will be placed over the area.

As mentioned above we would like to take blood samples during the acute bout of exercise and after completion. Blood will be collected at three time points post-exercise (at the same time-points as muscle samples), which will allow us to analyse certain metabolites, of which glucose and triacylglerides will be included. The blood will immediately be placed in special tubes for later analysis.

Muscle sample (biopsies): Most of the changes with exercise are reflected by physiological and metabolic changes in the working muscle. To look at these factors we need to have a small sample of your thigh muscle, the sample is taken using a procedure known as a "muscle biopsy". The biopsy procedure consists of anaesthetising the skin, making a very small incision in the skin and inserting a fine-needle (for three of the samples, and then a "tweezer-like" device to extract a larger sample - still only ~ 10th of a gram - for the larger sample), which will take a very small sample of muscle, approximately 20mg (50th of a gram). Probably the most painful part (if you feel anything) is the injection of local anaesthetic, which may sting for about 10 seconds. Thereafter the sensations are of pressure in your muscle. The preparation time, cleaning the skin, injecting the anaesthetic (and waiting for it to work), takes about 15 minutes, whilst the actual biopsy is all over in about a second. Afterwards, the very small incision in the skin is closed with Steristrips, and a plaster and a sterile dressing taped over the site. You need to keep the site dry for about four days to allow the cut to heal. When the anaesthetic wears off you may feel the biopsy site to be rather tender and there may be some bruising. But in general a muscle biopsy is very well tolerated and there have been lots of studies where participants have had biopsies in the middle of races and then carried on. The muscle itself recovers rapidly and the biopsy will have no effect on your muscle strength. The

biopsy will be taken by a qualified doctor who is very experienced in this type of procedure. Each whole procedure will take approximately 20 minutes.

You should not have a biopsy if you have ever had an adverse reaction to a local anaesthetic (such as when having dental treatment) or if you have a tendency to bleed excessively. After the biopsy you should keep an eye on the cut and check it is healing properly. In the unlikely event that it becomes red and inflamed you should immediately see your GP and keep us informed.

Muscle biopsy and blood storage: Both your blood and muscle samples will be anonymously labelled/coded and stored at $-80^{o}C$, which will enable us to perform tests of importance for this study, like: genetic analysis, muscle fibre type identification, and metabolite analysis. We will only be analysing genes and metabolites, which are of interest and have previously been identified in other studies, which may be relevant in metabolic fitness.

I have read and understood the nature of the tests and procedures, including the blood samples and muscle biopsies (and storage, and subsequent analysis) and what they involve. I give my consent to taking part in the study but realise that I can withdraw from the study at any time without having to give an explanation.

Signed…… Date……

Name (Print)……..

Witnessed……

Name (Print)……..

If you have any questions about the study as a whole or about individual tests or procedures, now or at any time in the future, please contact David Vaughan (0161-2475457) or Professor Marin Flueck (0161-2475417).

All data obtained will be treated as strictly confidential. You will also have the option to receive specific advice regarding nutrition and exercise based on our findings. We are more than happy to talk to you about any results and what they may imply. At the end of the study we will compile a short report for all the participants to keep you informed about the contribution you will have made to advancing knowledge.

Thank you for your interest and help.

Participant Statement:

I fully understand what is involved in taking part in this study. Any questions I have about the study, or my participation in it, have been answered to my satisfaction. I understand that I do not have to take part and that I may decide to withdraw from the study at any point without prejudice. I have had my attention drawn to the document 'Ethical Regulations for the Use of Humans in Research'. My concerns regarding this study have been answered and such further concerns as I have during the time of the study will be responded to. It has been made clear to me that, should I feel that these Regulations are being infringed or that my interests are otherwise being ignored, neglected or denied, I should inform the Chair of the Ethics Committee of the Department of Exercise and Sport Science, Manchester Metropolitan University, Hassall Road, Alsager, Cheshire, ST7 2HL who will undertake to investigate my complaint.

Signed ..... Date .....

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Appendix 2: Publications, Proceedings and Communications

Publications:

Martin Flueck, **David Vaughan**, and Hakan Westerblad. Linking genes with exercise: where is the cut-off? European Journal of Applied Physiology (2010): 110; 1095-1098.

Abstract: Studies on gene-phenotype associations are a popular theme in exercise physiology. This editorial follows up on the current limitations in this quest with regard to the identification of mechanistically important relationshipds.

Manuscripts submitted and in preparation:

David Vaughan, Felicitas A Huber-Abel, Franziska Graber, Hans Hoppeler, and Martin Flueck, The Angiotensin Converting Enzyme Insertion/Deletion Polymorphism Alters the Response of Muscle Energy Supply Lines to Exercise - *submitted to the European Journal of Applied Physiology*

Conference proceedings:

Michael Brogioli, **David Vaughan**, Wouter Eilers, Sarah Waldron, Martin Flück, Glucose oxidation during endurance work is reduced in ACE genotypes lacking the I-allele, [4]. Congress of the Sportwissenschaftliche Gesellschaft der Schweiz (2012), Eigenössische Hochschule für Sport, Magglingen, Switzerland.

Introduction: Oxidation of blood borne nutrients carbohydrate and triglyceride importantly drive endurance performance[1]. The vasoconstrictor angiotensin 2 may play an important role in the underlying metabolic control. This is indicated by the improved glucose disposal with pharmacological inhibition of angiotensin 2 production by angiotensin converting enzyme (ACE; [4]) and elevated serum angiotensin 2 levels post exercise[3]. We hypothesized that modulated nutrient supply explains the reduced endurance performance in subjects with genetically enhanced levels of the major vasoconstrictor angiotensin 2 due to the presence of a silencer region (the I-allele) in the ACE gene[2].

Methods: Amateur endurance runners were screened for the I-allele with polymerase chain reaction in mucosal samples. Serum glucose and triglycerides were measured using a cardiocheck (brand) in venous blood samples before and immediately after a Marathon race in Chester (UK). A subset of subjects reported to the laboratory for the apprehended investigation of serum glucose and triglycerides in relation to serum angiotensin 2 (ELISA, Sp-Bio) and respiration exchange ratio (Cortex system) during an exhaustive single leg endurance test on a stationary bicycle ergometer. The protocol consisted in 25 minute at 60% peak power output followed by an increase of the intensity until exhaustion.

Results: Serum glucose but not triglyceride concentration after the Marathon was significantly higher in ACE genotypes lacking the I-allele vs. those with the I-allele (7.6 vs. 4.4 mM, n=4 vs. 12; p=0.0001, T-test). No difference between the race time of ACE genotypes lacking the I-allele vs. those with the I- allele was found (214 vs. 201 min, p=0.703, n=5 vs. 13, T-Test). When we raised the threshold of running time, no athletes that ran under 3 hours showed a genotypes lacking the ACE I-allele (p=0.03, sign test, n=5). Fold changes in glucose concentration after 30 minutes single leg bicycle exercise were also higher in ACE genotypes lacking the I-allele (1.19 vs. 1.06, p=0.07, n=2 vs. 6, T-test) which demonstrated elevated angiotensin 2 levels at rest.

The changes in glucose correlated to average respiration exchange ratio (r=0.70; p=0.08; n=7) and changes in serum angiotensin 2 (r=-0.846; p=0.008; n=8) at the end of the exhaustive ergometer exercise.

Discussion/Conclusion: Altered genetic drive in the producer of the major vasoconstrictor, angiotensin 2, explains 50% of the variability in glucose oxidation with endurance work and appears to be a handicap to achieve competitive race time during an extensive run. The findings imply that ACE genotypes lacking that the I-allele show inhibited uptake of blood borne substrates in working muscle.

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gene accounting for half the variance of serum enzyme levels. The journal of clinical investigation, 86, 1343–1346.
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Angiotensin Converting Enzyme Exerts System Control Over Fuel Handling In Skeletal Muscle

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Using a genetical metabolomics approach we assessed whether altered energy supply in locomoter muscle underlies the elevated aerobic performance of human genotypes containing a silencer region (I-allele) of the major regulatory enzyme of vasoconstriction, angiotensin converting enzyme (ACE).

Extensor muscle, m. vastus lateralis, of ACE-II/ID genotypes holding the I-allele demonstrated a trend for elevated capillarity compared to ACE-DD counterparts lacking the I-allele (311.0 vs. 279.7 mm-2, n=20, p=0.10). In untrained subjects, maximal oxygen uptake during bicycle exercise was lower in ACE-DD genotypes (44.9 vs. 47.8 ml O2/min/kg). Exhaustive aerobic exercise selectively reduced low density lipoproteins in serum of ACE-II/ID genotypes (–14%) but was spared in ACE-DD (+6%, p=0.25). By contrast, non-polar metabolites in exercised muscle,

comprising tentatively identified LDL-derived glycerophosphocholine species, were depleted in ACE-DD genotypes (q=2.8%; statistical analysis of microarrays). The interaction effect of exercise and genotype on lipidic muscle metabolites was maintained in trained subjects (p=0.03, ANOVA). The observations indicate that elevated import of serum lipids into exercised muscle underlies the enhanced aerobic exercise performance of I-allelic genotypes for this major checkpoint of vascular perfusion.

Poster Communications

University of Manchester (2010) Proc Physiol Soc 19, PC175

Angiotensin converting enzyme exerts system control over fuel handling in exercising skeletal muscle

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Blood-borne nutrient supply is critical to fuel muscle contraction and limits exercise performance. A silencer region (I-allele) within intron 16 of the gene for the major regulatory enzyme of vasoconstriction, angiotensin converting enzyme (ACE), is implicated in phenotypic variation of human performance and its trainability. The muscular mechanisms contributing to ACE-modulated exercise performance is not known. We hypothesized that modified substrate supply and aerobic metabolism in skeletal muscle underlies the modulation of maximal aerobic exercise performance by the genetic silencing of ACE expression. This was exposed using a genetical-metabolomics approach. Human subjects, who gave informed consent, were recruited to characterize ACE-dependence in muscle makeup and muscle’s metabolic response to exercise. Aerobic performance was assessed by ergospirometry. Muscle biopsies were collected (under lidocaine as local anaesthetic), from the major extensor muscle

vastus lateralis, before and 30 min after a bout of maximal aerobic exercise at a standardized intensity on an ergometer (Jaeger). Biopsies of untrained healthy subjects (n=20) were subjected to transcript profiling and ultra-structural analysis, carried out as described (Schmutz et al 2010). Muscle metabolites of untrained (n=10) and trained (n=10) subjects were extracted from cryo-sectioned samples into a polar (methanol) and non-polar (chloroform) phase and assessed by liquid chromatography - mass spectrometry (LC-MS) and direct infusion mass spectrometry (DIMS), respectively, as described in (Dunn et al 2008; Brown et al 2009). ACE-DD genotypes demonstrated elevated muscle capillarity (+17.8%; $p<0.05$) and increased expression (assessed by significance analysis of microarray - SAM) of transcripts related to vascular remodelling and lipid metabolism compared to ACE-II/ACE-ID genotypes (q-value = 2.39; fold change 1.01 - 2.08). Oxidation of lipid substrates (as assessed by respiration exchange ratio; -30%; p=0.30, ANOVA) and levels of non-polar, but not polar, metabolites were reduced with exercise in the ACE-DD genotype lacking the I-allele (-33%, p=0.06, repeated ANOVA). The selective depletion of the non-polar metabolite class was preserved in trained subjects (-34%, p=0.03). Serum LDL was selectively reduced (-14%, p=0.03) in the genotypes with the I-allele. The observations demonstrate a clear difference in whole-body substrate utilisation between the investigated ACE-genotypes during maximal endurance exercise, which relates to muscle metabolite levels. A mismatch between vascular delivery and myocellular turnover of non-polar metabolites upon exercise is suggested to underlie the different trainability of genotypes for the major checkpoint of vascular perfusion, ACE (Montgomery et al 1998).

University of Dublin (2009) Proc Physiol Soc 15, C34

Aerobic Fitness in Humans is Under System Control by The Angiotensin 2 Pathway

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Background*:* Blood supply is critical to fuel muscle contraction and limits exercise performance. The presence of a silencer sequence in intron 16 (insertion, I) in the ACE (angiotensin converting enzyme) gene, which is the enzyme that converts the vasoconstrictor angiotensin (Ang) into its active form Ang2, has been associated with

enhanced endurance capacity (Montgomery et al., 1998 and Scanavini et al., 2002). However, conflicting evidence exists on the association of ACE genotypes with regard to the presence (or absence) of the I sequence and human performance (Rankinen et al. 2000, Amir et al., 2007 and Zhao et al., 2003).

***Hypothesis**:* Adaptations of capillary supply lines in muscle contribute and explain the altered endurance phenotype in ACE genotypes, with an ACE insertion, due to reduced ACE gene expression in muscle.

Methods: Participants were untrained healthy medical students (n=12) recruited from the University of Berne. Aerobic performance was measured and muscle biopsies collected, under local anaesthetic (lidocaine), from the *Vastus lateralis* muscle. Ultra-structure and gene expression were quantified in biopsies with morphometry and cDNA microarrays. DNA was extracted and the ACE genotype identified post-hoc with specific primers.

Results: The investigated participants fell into two categories: DD and ID. The DD genotype vs. the ID genotype demonstrated increased mRNA expression of ACE and Ang2 receptors, elevated mitochondrial density, capillary density, and reduced fiber size (see fig. 1). Maximal oxygen uptake during bicycle exercise in the DD genotype was elevated by 27% and correlated positively and negatively, respectively, with mitochondrial volume density (r=0.65) and fiber area (r=-0.53) in the investigated knee extensor muscle.

Figure 1. Ultra-structural and mRNA differences between ACE DD v ID genotype

	Genotype	Mitochondria[a, z] (s.e.m.)	Capillary density[b] (s.e.m.)	Fiber area[c] (s.e.m.)	ACE mRNA[d] (s.e.m.)	AGTR1[e] mRNA[d] (s.e.m.)	AGTR2[f] mRNA[d] (s.e.m.)
Mean	DD (n=7)	4.8 (0.4)	869.1 (38.6)	3060.0 (152.7)	19.5 (1.9)	9.1 (3.4)	9.7 (3.2)
	ID (n=5)	3.3 (0.3)	687.0 (55.0)	3753.5 (276.8)	15.7 (4.5)	4.9 (1.5)	5.3 (1.5)
% change		+47	+27	-18%	+25%	+86%	+83%
P[g]/Q[h] value		0.04	0.01	0.04	15.2	9.9	9.9

[a]Per fiber volume, [b]mm/mm^3 of fiber volume, [c]μm^2, [d]normalised to total (x10^{-4}) mRNA per array, [e]angiotensin receptor-1, [f]angiotensin receptor-2, [g]honest significant difference for unequal number, [h]statistical analysis of microarrays (SAM), [z]only 4 samples analysed

Discussion & Conclusion: The investigated, not-specifically trained population demonstrated a shift towards a muscle phenotype with improved substrate supply and aerobic metabolism. This points out that expressional alterations in the local Ang2 signalling system intervene in the manifestation of an endurance phenotype via the modulation of capillarity and aerobic capacity in locomotor muscle.

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